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Organoids Model Distinct Vitamin E Effects at Different Stages of Prostate Cancer Evolution

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ABSTRACT

Prostate cancer (PCa) is the most common non-cutaneous cancer among U.S. men. Lack of effective treatments for advanced disease make it a significant public health concern. However, PCa's long natural history makes it an excellent target for prevention approaches that reduce overtreatment of indolent disease, treatment related morbidity, and mortality. Oxidative stress has long been linked to prostate carcinogenesis. This fueled interest in the use of antioxidant supplements to inhibit, reverse or slow precancerous events or disease progression (chemoprevention). Analyses of the Alpha-Tocopherol, Beta Carotene (ATBC) prevention and the Nutritional Prevention of Cancer (NPC) trials showed a reduction in risk of PCa as a secondary end point after supplementation with the antioxidants, alpha tocopherol and selenium respectively.

Additionally, epidemiological and preclinical data suggest an anti-tumorigenic role for vitamin E and selenium against PCa. However, the selenium and vitamin E Cancer Prevention Trial (SELECT), testing the efficacy of vitamin E and/or selenium on reducing PCa incidence in 35,533 healthy men found vitamin E to be associated with an increased risk of PCa while selenium was not efficacious. The work in this dissertation focused on understanding SELECT's negative outcome. We hypothesized that the lack of clinical translation for the preclinical *in vitro* data was because the latter were derived by testing vitamin E and selenium on advanced PCa cell lines grown in non-physiologic two dimensional cell cultures.

Further, we hypothesized that the more physiologic three-dimensional cultures would yield more clinically relevant data and that the outcome of antioxidant treatment would depend on the stage

of the cancer. To test this, we studied the effects of vitamin E and selenium on a continuum of prostate carcinogenesis from benign, premalignant to malignant cells in three-dimensional organoid cultures which mimic *in vivo* prostate gland architecture. We found that while the supplements decreased proliferation and induced cell death in cancer (LNCaP) organoids, they had no effect on benign organoids derived from normal primary human prostate epithelial cells. This confirms that antioxidants have a different impact on different stages of cancer.

Additionally, vitamin E but not selenium alone or in combination, enhanced cell proliferation and survival in premalignant (RWPE-1) organoids, recapitulating the SELECT results. Relative to vehicle, the vitamin E treated premalignant organoids had low ROS levels and more luminal filling. Furthermore, microarray analysis revealed downregulation in the expression of integrins, glucose transporters and glycolytic enzymes in the vitamin E treated premalignant organoids, suggesting matrix detachment and metabolic alterations. Accordingly, detached RWPE-1 cells treated with vehicle had low ATP levels due to diminished glucose uptake and glycolysis.

However, treating detached RWPE-1 cells with vitamin E rescued ATP by activating fatty acid oxidation (FAO). FAO inhibition abrogated vitamin E's ATP rescue in detached RWPE-1 cells and diminished survival of the inner matrix deprived cells, restoring the normal hollow lumen morphology in vitamin E treated organoids. Organoid models therefore clarified the paradoxical findings from SELECT. These findings demonstrate that vitamin E promotes tumorigenesis in the early stages of prostate cancer evolution by promoting cell survival in matrix deprived cells by activating FAO.

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LIST OF ABBREVIATIONS

18F-FDG – 18F-fluorodeoxyglucose

2D – Two-dimensional

2DG – 2-Deoxy-D-glucose

3D – Three-dimensional

4-HNE – 4-Hydroxynonenal

5ARIs – 5α-Reductase inhibitors

8-OHdG – 8-Hydroxy-2-Deoxy Guanosine

A.U. – Arbitrary Units

AA – African-American Men

ABCA1 – ATP Binding Cassette Subfamily A Member 1

ACC – Acetyl-CoA carboxylase

ACMG – American College of Medical Genetics and Genomics

ACOX3 – Acyl-CoA Oxidase 3, Pristanoyl

ACS – Acetyl-CoA Synthase

ADT – Androgen Deprivation Therapy

ALL – Acute Lymphoblastic Leukemia

ALOX5 – Arachidonate 5-lipoxygenase

AMACR – α-methylacyl-CoA racemase

AML – Acute Myeloid Leukemia

AMPK – 5' AMP-activated protein kinase

ANOVA – Analysis of Variance

APL – Acute promyelocytic leukemia

AR – Androgen Receptor

AR-V7 – Androgen-receptor splice variant 7

ASNS – Asparagine Synthetase

ATBC – Alpha-Tocopherol Beta Carotene Clinical Trial

ATP - Adenosine triphosphate

AUA – American Urological Association

BPE – Bovine Pituitary Extract

BPH – Benign Prostatic Hyperplasia

BrDU – Bromodeoxyuridine

CACT – Carnitine-acylcarnitine translocase

CAFs – Cancer-associated Fibroblasts

CAT – Catalase

CAVs – Caveolins

CCCP – Carbonyl cyanide m-chlorophenyl hydrazine

CDEs – CAF-derived exosomes

CDRs – Cancer Detection Rates

CNV – Copy Number Variation

CPT I – Carnitine palmitoyltransferase I

CRPC – Castration Resistant Prostate Cancer

CRPC – Castration Resistant Prostate Cancer

Cyclo-oxygenase – COX

D2HG – D-2-Hydroxyglutarate

DAG – Diacylglycerol

DAPI – 4′,6-diamidino-2-phenylindole

DCA – Dichloroacetate

DEC1 – Deleted in esophageal cancer 1

DEGs – Differentially Expressed Genes

DHAP – Dihydroxyacetone phosphate

DHT – 5α-dihydrotestosterone

DNA – Deoxyribonucleic Acid

DRE – Digital Rectal Examination

EA – European-American Men

EAAC1 - excitatory amino-acid carrier 1

EBRT – External beam radiation therapy

ECAR – Extracellular Acidification Rate

ECM – Extracellular matrix

EGF – Epidermal Growth Factor

ELOVL7 – Elongation of very long chain fatty acids protein 7

ER – Endoplasmic Reticulum

ES – Enrichment Score

ETC – Electron Transport Chain

FA – Fatty Acid

FADH2 – Flavin adenine dinucleotide

FAO – Fatty Acid Beta Oxidation

FASN – Fatty Acid Synthase

FBS – Fetal Bovine Serum

FDA – Food and Drug Administration

FDG-PET – 18-Fluoro-deoxyglucose positron emission tomography

FDR – False discovery rate

FPC – Familial Prostate Cancer

FPP – Farnesyl pyrophosphate

GATK – Genome Analysis ToolKit

GEMMs – Genetically engineered mouse models

GGPP – Geranylgeranyl pyrophosphate

GLS – Glutaminase

GLUT – Glucose Transporter

GnRH – Gonadotropin-releasing hormone

GPI - Glycosylphosphatidylinositol

GPX – Glutathione peroxidase

GSEA – Gene Set Enrichment Analysis

GSH – Glutathione

GSK3 – Glycogen synthase kinase 3

GST – Glutathione S-transferase

GWAS – Genome-wide Association Studies

H&E stain – Hematoxylin and eosin stain

HBP – Hexosamine Biosynthetic Pathway

HGMD – Human Gene Mutation Database

HGPIN – High Grade Prostatic Intraepithelial Neoplasia

HIFU – High-intensity focused ultrasound

HK2 – Hexokinase 2

HMGCR – HMG-CoA reductase

HPB – Hexamine Biosynthetic Pathway

HPC – Hereditary Prostate Cancer

HPV – Human papillomavirus

HSP – Heat Shock Proteins

ICAM 1 – Intercellular Adhesion Molecule 1

IDH1/2 – Isocitrate dehydrogenase 1 and 2

IMS – Intermembrane space

IRB – Institutional Review Board

IU – International Units

LBD – Ligand Binding Domain

LD – Lipid Droplets

LGPIN – Low Grade Prostatic Intraepithelial Neoplasia

LH – Luteinizing hormone

LHRH – Luteinizing hormone-releasing hormone

LOA – Loss of Attachment

LOH – Loss of heterozygosity

LP – Lateral Prostate

mAAT – Mitochondrial aspartate aminotransferase

MCM – Minichromosome maintenance protein complex

mCRPC – Metastatic Castration Resistant Prostate Cancer

MCTs – Monocarboxylate transporters

MMPs – Matrix metalloproteinases

MNU – N-methyl N-nitroso Urea

mPCa – Murine Prostate Cancer

MSA – Methylseleninic acid

MSC – Methyl selenocysteine

MSigDB – Molecular Signatures Database

MTHFD2 – Bifunctional methylenetetrahydrofolate dehydrogenase 2/cyclohydrolase

NAC – N-Acetyl-Cysteine

NADH – Nicotinamide adenine dinucleotide

NADPH – Nicotinamide adenine dinucleotide phosphate

NCI – National Cancer Institute

NES – Normalized enrichment score

NMU – N-Nitroso-N-methylurea

NPC – Nutritional Prevention of Cancer Clinical Trial

NSCLC – Non-small cell lung cancer

OCR – Oxygen Consumption Rate

OCT – Organic Cation Transporter

OXPHOS – Oxidative phosphorylation

PCa – Prostate Cancer

PDH – Pyruvate Dehydrogenase

PDK – Pyruvate Dehydrogenase Kinase

PHDGH – Phosphoglycerate dehydrogenase

PHDs - Prolyl hydroxylases

PIA – Proliferative Inflammatory Atrophy

PIN – Prostatic Intraepithelial Neoplasia

PIP3 – Phosphatidylinositol (3,4,5)-trisphosphate

PKC – Protein Kinase C

PKM2 – Pyruvate kinase muscle isozyme M2

PLND – Pelvic lymph node dissection

Poly-HEMA – Poly (2-hydroxyethyl methacrylate)

PPP – Pentose Phosphate Pathway

PrE – Primary Epithelial Cells

PrEC – Prostate Epithelial Cells

PrEGM – Prostate Epithelial Cell Growth Medium

PRX – Perodoxin

PSA – Prostate specific antigen

PUFAs – Polyunsaturated fatty acids

qRT-PCR - Quantitative Reverse Transcription Polymerase Chain Reaction

RB – Retinoblastoma protein

REDUCE – REduction by DUtasteride of prostate Cancer Events

RMA – Robust Microarray Analysis

RNA – Ribonucleic Acid

RNS – Reactive Nitrogen Species

ROS – Reactive Oxygen Species

RP – Radical Prostatectomy

RT – Radiation Therapy

SCD1 – Stearoyl-Coenzyme A desaturase-1

SD – Standard Deviation

SDH – Succinate dehydrogenase

SELECT – Selenium and Vitamin E Cancer Prevention Trial

SeMet – Selenomethionine

SNP – Single Nucleotide Polymorphism

SNV – Single Nucleotide Variant

SOD – Superoxide dismutase

SPC – Sporadic Prostate Cancer

SRE – Sterol Regulatory Element

SREBP – Sterol regulatory element-binding proteins

TBARS – Thiobarbituric acid reactive substances

TCA Cycle – Tricarboxcylic Acid Cycle

TET enzymes – Ten-eleven Translocation Enzymes

TOB – RRR-alpha-tocopheryloxybutyric acid

TRAMP – Transgenic adenocarcinoma of the mouse prostate

TRs – Thioredoxin Reductases

TXNIP – Thioredoxin interacting protein

UIC – University of Illinois at Chicago

uPAR – Urokinase-type plasminogen activator receptor

USPSTF – United States Preventive Services Task Force

VEBSA – RRR-alpha-tocopheryloxybutyl sulfonic acid

VES – Vitamin E Succinate

VITAL – VITamins And Lifestyle cohort study

Zip1 – Zinc Transporter 1

DEDICATION

To my mum Pauline Nyachomba Njoroge and the memory of my dad, Joseph Njoroge Kimani. I would not have done this without your unconditional support. I miss you so much dad; your example will forever inspire my life experiences.

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Njoroge RN, Unno K, Zhao JC, Naseem AF, Anker JF, McGee WA, Nonn L, Abdulkadir SA (2017). *Organoids model distinct Vitamin E effects at different stages of prostate cancer evolution.* Sci Rep.7 (1):16285.

CHAPTER 1: INTRODUCTION

Prostate Cancer Epidemiology

Prostate cancer (PCa) is the most common non-skin cancer in American men which is expected to be diagnosed in the lifetimes of one out of nine American men.[1,2] In 2018 alone, there were an estimated 164,690 new cases and 29,430 PCa associated deaths.[3] Age is the most significant risk factor for PCa development. PCa's prevalence is roughly 35% in men aged 60 – 69 years and 46% in men aged 70 – 81.[4] The median age of diagnosis is 66 years with the majority of cases being diagnosed between the ages of 65 – 74.[5]

PCa is a bigger health problem in developed countries where there is a bigger proportion of elderly men with 15% of all male cancers being PCa related compared to developing countries where just 4% is the case.[6] However, some of the differences in disease epidemiology are attributable to screening, availability of early detection and treatment options.[1] In the U.S., the incidence of PCa is 1.6 times higher among African American men compared to Caucasian men.[7]

Prostate Cancer Risk Factors

Risk factors that have inconsistently been associated with PCa include obesity, diet, metabolic syndrome, smoking, physical inactivity and exposure to heavy metals and herbicides.[8-13] Age, race and a family history of PCa on the other hand are well-established risk factors for developing the disease.[14,15]

Age

PCa has the one of the strongest associations between age of any human cancer.[16] Though PCa is rarely diagnosed before 40 years of age, the incidence rises sharply thereafter.[17] Between 2011 and 2015, the PCa incidence rates per 100,000 persons for men aged 35 – 39 years, 60 – 64 years and 65 – 69 years were 0.8, 423.2 and 651.7 respectively.[17] Elevated oxidative stress and diminished antioxidant defenses with advancing age have been proposed to explain the association between age and PCa.[18] Inflammation and dysregulation of androgen regulated redox homeostasis are thought to be major contributors of prostatic oxidative stress.[18] The prostate gland is prone to chronic inflammation probably due to its anatomical proximity to the urinary tract.

Though the exact cause of prostatic inflammation is unknown, waste stimuli from urine reflux, injury from mechanical obstruction by uric acid crystals, as well as infection have been implicated.[19] Whatever the cause, histological inflammation has been detected in the majority of prostate tissues. In the REDUCE (REduction by DUtasteride of prostate Cancer Events) trial testing the efficacy of dutasteride in reducing the risk of PCa in men with a negative PCa biopsy, 78% of the enrollees were found to have chronic histologic inflammation.[20,21]

In fact, oxidative stress from chronic inflammation mediated by cyclo-oxygenase (COX) has led to PCa prevention trials using asprin a nonsteroidal anti-inflammatory drug or COX-2 inhibitors.[22-24] Other factors that increase PCa risk that are impacted by age include cholesterol metabolism and obesity, levels of sex hormones and increasing accumulation of genetic and epigenetic alterations.[25] However, the mechanistic links between aging and cancer development in general remain under investigation, it is hoped insights from the former could be leveraged in cancer therapies.[26]

Race

PCa epidemiology displays significant racial and ethnic differences. The age adjusted PCa incidence rates per 100,000 U.S. males of all ages between 2011 – 2015 were 178.3 for African American men (AA), 105.7 for European-American men (EA), 91.8 for Hispanics, 59.1 for Asian/Pacific Islanders and 54.8 for Native Americans.[17] The corresponding PCa related mortality rates per 100,000 U.S. males of all ages were 39.9 for blacks, 18.2 for white, 16.2 for Hispanics, 14.7 for Native Americans and 8.8 for Asian/Pacific Islanders.[17] Therefore, in the U.S., black men are at 1.7 fold higher risk of a PCa diagnosis and are twice as likely to die from PCa.[17] In addition to the high incidence and mortality rates among AA men, they also have a higher proportion of early onset disease (≤ 55 years at diagnosis) compared to other racial groups.[27]

In one study, (n > 12,000) 8.3% AA men were less than 50 years of age at time of diagnosis compared to 3.3% EA men.[28] In contrast, Native Americans and Asian/Pacific islanders have the lowest incidence rates and PCa mortality rates, respectively among U.S. males.[17] The reasons for these disparities remain unclear. Studies have found that AA men typically present with higher PSA levels and more advanced PCa at diagnosis compared to other racial groups.[29-31] However, in the Prostate Cancer Outcomes Study, the higher risk for advanced PCa in AA men did not change after adjustment for socio-economic factors and clinical variables.[29] A different study reported that even when diagnosed early and at a similar pathological stage, AA still had a slightly higher risk for biochemical recurrence than EA men.[32] However, because ethnicity is closely linked to genetic factors, it is thought that there is a molecular basis for the racial

differences in incidence and PCa outcomes including mutations, genetic polymorphisms and epigenetics.[33,34]

For example, the androgen receptor (AR) is important in prostate development but also in prostate carcinogenesis. It has been shown that there are ethnic differences in the distribution of polymorphisms that regulate AR activity.[35] Polymorphic cytosine, adenine and guanine trinucleotide repeats (CAG)n in exon 1 of the AR gene encoding poly-glutamine are thought to regulate AR activity with shorter alleles conferring increased AR activity.[35] AA men have significantly shorter (CAG)n repeat length than CA men with increased androgen sensitivity.[35]

Family history

Compared to men in the general population, first-degree relatives of men with PCa are at twice or thrice the risk of developing the disease, and those with two or more affected first degree relatives have 5 – 11 fold increased risk.[36-38] The familial PCa risk is compounded in early onset cases with first degree male relatives of men diagnosed at ≤ 60 years being at a higher risk compared to men whose relatives were diagnosed at > 60 years.[33,39] Familial clustering of PCa can result from inherited susceptibility, lifestyle choices, environmental factors and their interactions.[40]

The contribution of genetic factors to PCa familial aggregation is evidenced by Nordic twin registries analysis showing that monozygotic twins have 50% higher risk than dizygotic twins.[41,42] In fact, genetic factors may be involved in up to 42% of all PCa cases making it perhaps the most heritable cancer.[41] Moreover, ethnic and racial differences in PCa whose incidence is highest among African American men and lowest among Americans of Asian

ancestry further support the role of genetic factors in PCa carcinogenesis.[3] However, differences in PCa incidence rates between native and migrant populations suggest that environmental and lifestyle factors play a role in disease risk.[43]

Based on family history, PCa can be categorized as familial (FPC), sporadic (SPC) or heritable (HPC).[44] SPC cases have no prior family history, and they constitute the majority of all PCa cases at 75 – 85%.[44] Both FPC and HPC have a positive familial history, however, while FPC describes general PCa aggregation in families, HPC specifically describes a subset of FPC that displays a pattern of Mendelian inheritance of susceptibility gene(s).[40] FPC makes up 10-20% of the cases and hereditary prostate cancer (HPC) between 5 – 10%.[40] However, despite these distinctions, sporadic PCa also has a germline genetic component.[45] Moreover, HPC, FPC and SPC are said not to differ clinico-pathologically expect for the 6 to 7 years earlier presentation of HPC cases.[40,46,47]

Genetic susceptibility

Genetic predisposition to a disease can be attributed to rare (highly penetrant) mutations, genetic variants conferring moderate to low risk or a combination of both.[48] Segregation analysis in diverse populations suggest that familial aggregation of PCa follows autosomal dominance, recessive, X-linked or multi-factorial inheritance patterns.[49-57] However, despite strong evidence for genetic predisposition to PCa, results from linkage studies on families with multiple cases to pinpoint susceptibility genes have not been consistently replicated with the exception of HOXB13.[58] Other debated genes include HPC2/ELAC2, HPC1/RNASEL, MSR1, PCAP,

CAPB, BRCA2 and BRCA1 but their true association with PCa risk needs further validation.[48,59-68]

The difficulty in identifying consistent susceptibility loci across wide populations has been attributed to genetic heterogeneity and the involvement of multiple incompletely penetrant alleles.[69] Additionally, genome-wide association studies (GWAS) have identified over 76 Single Nucleotide Polymorphisms (SNPs) associated with PCa.[48] It is said that 28.4% relative risk of FPC can now be attributed to common variants identified by GWAS.[70] Another 6% might be explained by rarer but higher risk variants such as those in the BRCA2 and HOXB13 genes. [70]

Prostate anatomy

The prostate is an exocrine gland located near the bladder (Fig 1).[71] Its secretions are necessary for male fertility and they are enriched in Zn2+, citrate, and Kallikrein-related peptidases.[72] Prostatic secretions empty through ducts into the prostatic urethra where they make up part of the seminal fluid.[71] The prostate gland has three distinct anatomical zones that differ in location, histology and disease propensity (Fig 1).[73] The central zone borders the ejaculatory ducts and it runs from the base of the bladder tapering off at the verumontanum.[74]

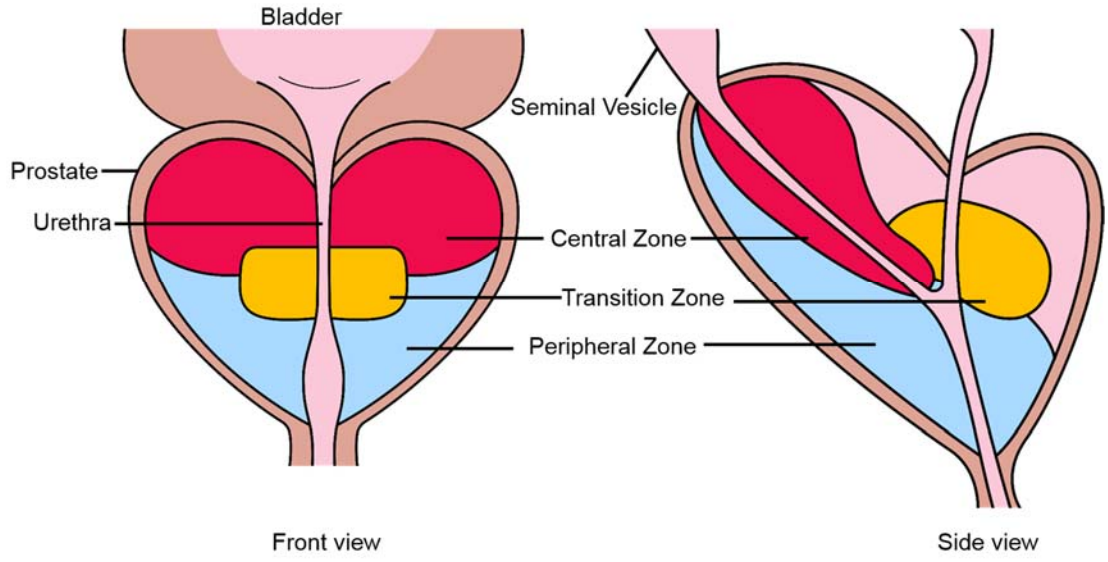

Figure 1. Zonal anatomy of the prostate gland. The human prostate is divided in to three glandular zones; the central, transition and peripheral zones.

The peripheral zone which makes up the bulk of the prostatic volume contains the prostatic ducts, and it surrounds the central zone stretching down to the prostate apex.[74] The periurethral transition zone is situated next to the proximal urethra, and it borders the central and peripheral zones.[74] While benign prostatic hyperplasia (BPH), a common nonmalignant condition develops in the transition zone, most PCas develop within the peripheral zone.[73] Histologically, the prostate is a pseudostratified epithelium of luminal and basal epithelial cells and rare neuroendocrine cells.[73] The luminal layer is made of polarized columnar luminal epithelial cells that produce prostatic secretions and highly express AR and low molecular weight cytokeratins 8/18, (CK8+/p63-/AR+).[73]

The basal cell epithelial layer separates the luminal cells from the stroma and expresses high molecular weight cytokeratins 5/14, p63 and much lower levels of AR, (CK5+/p63+/AR-).[73] The

prostate epithelium also contains cells that express both basal and luminal markers that are termed, transiently amplifying "intermediate cells."[71] The neuroendocrine cells express synatophysin and chromogranin A and they are thought to secrete factors necessary for luminal cell growth.[71] The prostate epithelium is surrounded by a stromal compartment with several cell types, including smooth muscle cells, myofibroblasts, endothelial cells among others.[71]

Prostate cancer pathogenesis

Precursor lesions of prostate cancer

Prostatic carcinogenesis is believed to proceed along a pathological and morphological continuum that evolves from benign glands through various premalignant stages and finally invasive disease (Fig. 2).[75] Proliferative inflammatory atrophy (PIA) and high grade prostatic intra-epithelial neoplasia (HGPIN) are some of the histologic lesions considered to be PCa precursors.[75] PCa lesions occur most commonly (80%) in the periphery zone of the gland with the rest occurring in the transition zone located in the periurethral region.[76] PIA, which is associated with chronic inflammation, is found in the peripheral zone and is characterized by focal glandular atrophy and postatrophic hyperplasia and epithelial cell proliferation.[77-79]

PIA lesions have been proposed to be precursors of prostatic adenocarcinoma directly or indirectly by progressing to PIN.[79] This notion is supported by the common presence of PIA near HGPIN and PCa foci.[77-79] Like PIA, PIN lesions are also found primarily in the peripheral zone, and they histologically range from the more normal low-grade PIN (LGPIN) to the more abnormal high-grade PIN (HGPIN).[80] Of the PINs, HGPIN, (grade 2–3) is considered to be a precursor for PCA development evidenced by the increased prevalence of HGPIN lesions in

prostates with carcinoma than those without.[81-83] PIN lesions are characterized by high proliferation rates, and they have some architectural, mutational and cytological alterations in common with early invasive carcinoma.[80,84,85]

PIN and PCa share mutations like SPOP, NKX3.1 and PTEN deletions, ERG (ETS transcription factor) rearrangements in addition to epigenetic aberrations like GSTP1 silencing.[86-90] The clonal relationship between HGPIN and PCa seems to depend on their promixity to each other.[91] HGPIN lesions share more genomic alterations with PCa foci like ERG rearrangement when they are in close proximity while those that lie further away do not harbor such aberrations.[91] However, HGPIN has low predictive value for subsequent PCa diagnosis.[92] Clinically, about 40% of men diagnosed with PCA within three years of HGPIN diagnosis.[81,93] HGPIN with specific aberrations like ERG overexpression may be more associated with a subsequent cancer diagnosis pointing to HGPIN heterogeneity.[94]

Prostate adenocarcinoma

Localized PCa is typically multifocal with multiple genetically distinct histologic foci.[73] Multifocal development of PCa is hypothesized to stem from a field effect where cell abnormalities occur beyond regions with morphologically evident tumor facilitating cancer initiation and concurrent development of HGPIN at multiple sites.[71,95] Studies to identify mechanisms underlying field cancerization in PCa are ongoing as these might yield molecular targets for prevention.[71] Interestingly, despite the phenotypic heterogeneity of metastatic PCas, multiple metastases from the same patient share similar genetic alterations indicating evolution

from a common clonal origin perhaps through acquisition of a selective advantage or through drug selection.[73]

Pathologically, 95% of prostate tumors are classified as adenocarcinomas in spite of the heterogeneity.[73] Histologically, the transition from PIN to adenocarcinoma is accompanied by several changes. Loss of basal cells is a hallmark of PCa, and most adenocarcinomas stain positively for α-methylacyl-CoA racemase (AMACR) a luminal marker that is upregulated in cancer but negatively for basal p63 and cytokeratin 5/14.[73] In addition, there is excess prostatic branching and abnormal cytology with nuclei and nucleoli enlargement.[71] However, though histologic changes can be detected in healthy men as young as 20 suggesting early disease initiation, PCa is not typically diagnosed until three or four decades later indicating slow progression.[73]

Mutations in Prostate Cancer

Like other cancers, PCa arises from the accumulation of genetic and epigenetic alterations in the prostate epithelium which co-evolve with alterations in cells in the extracellular microenvironment (Fig. 2).[96] The somatic alterations in PCa include single nucleotide variants (SNVs or point mutations), small insertions/deletions (indels) and genome rearrangements which result in large scale chromosomal structural alterations including deletions, duplications, inversions, insertions and translocations.[97,98] Point mutations result in protein amino acid changes (missense mutations) or truncations (nonsense mutations) while indels sometimes lead to changes in protein function (frameshift mutations).[99]

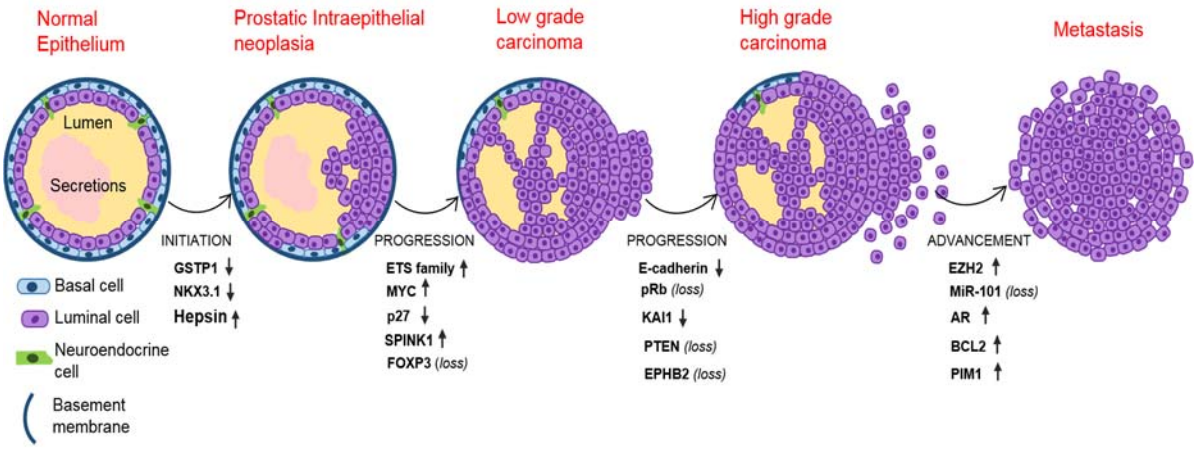

Figure 2. Model of prostate cancer initiation and progression. Stages of initiation and progression are shown, together with histological changes and concomitant genetic and epigenetic events that are likely to be significant at each stage.

Genome rearrangements can cause copy number variations (CNVs) which can amplify oncogenes or delete tumor suppressors and gene fusions that can confer gain of function and genome instability.[100] Relative to other malignancies, PCa has a low mutational frequency of 0.3–5 per Mb; point mutations are thought to play a minor role in prostate tumorigenesis.[101-104] The mutation frequencies of the top ten mutated genes in PCa are; TP53 (13%), PTEN (7%), SPOP (7%), AR (5%), FOXA1 (5%), LRP1B (4%), KM2TC (4%), KRAS (4%), FAT4 (4%), KMT2D (4%).[105] Recurrent mutations have also been detected in SPOP, FOXA1, TP53, ATM, PTEN, KDM6A, CDH1, APC, AR, ZFHX3, RB1, and MED12.[88,106]

In contrast, PCa has a high frequency of CNVs making structural chromosomal alterations the main drivers of PCa tumorigenesis.[103,104] The CNV burden, which defines the proportion of a tumor genome that is affected by structural changes, correlates with the Gleason score and

clinical outcomes like biochemical recurrence and PCa metastasis.[107,108] Genes mapped to chromosomal regions with CNVs in primary PCa include the oncogene MYC (8q24.21), and the tumor suppressors PTEN (10q23.31), NKX3.1 (8p21.2), RB1 (13q14.2), CDKN1B (12p13.1) and TP53 (17p13.1).[109] Overall, losses exceed gains and these can be either mono or bi-allelic, they could also be focal or affect large areas.[109]

PTEN (~15%) and CHD1 (~10%) are the most frequent homozygous deletions in PCa.[109] Others include BNIP3L, LRP1B (2q22.1), RB1, USP10, HTR3A, RYPB, MAP3K7, TP53 among others.[109] Per Knudson's "two-hit" hypothesis, loss-of-heterozygosity (LOH) of a tumor suppressor gene is required for tumor formation.[110,111] However, there is now ample evidence that loss of function is not always recessive, and the inactivation of a single allele can lead to tumor development through the reduction of protein dosage (haploinsufficiency) or in concert with other genetic and/or epigenetic changes.[112,113] In PCa, haploinsufficiency has been demonstrated for PTEN (10q23.31), NKX3.1 (8p21.2) and KLF5 (13q21).[114,115]

In the PCa genome, most gains tend to be hemizygous, though amplifications with more than two extra chromosomal copies have been reported primarily on 8q24 where MYC is located.[109,116] Additionally, analysis of clonal and subclonal genomic aberrations have allowed the temporal ordering of somatic genetic events in PCa. Early events tend to be clonal, and they are associated with cancer initiation and they can serve as disease biomarkers for early diagnosis or intervention.[117] Later events are subclonal, and they are associated with disease progression and cancer aggressiveness.[117] Early, clonal, events in PCa carcinogenesis include: NKX3-1 and FOXP1 deletions, TMPRSS2-ETS fusions, SPOP and FOXA1 mutations.[86,117]

However, ETS arrangements and SPOP mutations appear to be mutually exclusive, a trend that has been used for molecular subtyping of prostate tumors.[88,118] Late, sub-clonal events in PCa include CDKN1B and PTEN deletions and lesions in TP53, AR, and RB1.[86,117] In addition to genetic alterations, epigenetic alterations play an important role in PCa initiation and progression. For PCa, epigenetic alterations are an early event manifesting earlier than genetic changes and retained throughout the malignant progression.[90,119,120] Epigenetics refers to the different mechanisms that affect gene function and regulation without affecting the underlying DNA sequence and can be can be inherited transgenerationally.[121]

They include, DNA methylation, histone modifications, expression of non-coding RNAs, genomic imprinting among others. Some of the earliest epigenetic changes seen in PCa include DNA hypermethylation that silences GSTP1, APC and RASSF1A.[119,120] In contrast, DNA hypomethylation is associated with advanced disease, especially in metastasis.[122] DNA hypomethylation opens up chromatin and unmasks retrotransposable elements promoting genome instability.[121] The repertoire of epigenetic changes in PCa has recently been reviewed.[121]

Prostate cancer etiology

Though the mechanisms driving PCa development and progression are not well understood mechanisms under investigation include inflammation, androgen driven mechanisms, metabolism and oxidative stress.

Inflammation

Epidemiological, pathological, and molecular data support a causal role of chronic inflammation in prostate carcinogenesis.[73] Sources of prostatic inflammation include altered sex hormone

levels (androgenic activation of AR can increase the transcription of pro-inflammatory cytokines), bacterial and viral infections, physical trauma and dietary factors.[73,123] Prostatic inflammation is said to cause inflammatory cell infiltration and prostatic atrophy creating putative PCa precursor lesions termed proliferative inflammatory atrophy (PIA).[124] Immune cells in the prostate microenvironment increase the levels of inflammatory mediators like cytokines and reactive oxygen species (ROS), which damage DNA and increase cell proliferation.[125] PIA lesions, which proliferate probably in an attempt to repair cell damage, are characterized by oxidative stress and are hypothesized to progress to PCa through PIN.[124]

PIA and PIN lesions share some molecular alterations including loss of NKX3.1 and GSTP1 promoter methylation.[124] This suggests that inflammation from the prostate microenvironment may be involved in PCa initiation.[125] However, atrophic prostate epithelial cells might also secrete inflammatory mediators generating an inflammatory microenvironment causing neoplastic transformation.[125] Likewise, tumor cells support their growth by inducing an inflammatory microenvironment explaining how inflammation might play a role in PCa progression.[125] Prostatic inflammation has been linked mostly to lymphocytes and macrophages, and less often plasma cells and eosinophils.[126] Maspin inhibition and IKKα activation by prostate tumor infiltrating T cells, B cells and monocytic cells might promote metastasis.[125] Leukocyte and B cell prostate tumor infiltration after ADT therapy leads to CRPC by activating IKKα and STAT3.[125]

Suppression of T cell responses by myeloid-derived suppressor cells, which are recruited in response to increased IL6, has been linked to PCa progression and correlates with disease stage.[125] ROS, growth factors, chemokines and cytokines from infiltrating macrophages have

been shown to remodel tissue and lead to PCa progression in experimental models.[125] Tumor promoting pro-inflammatory cytokines in the prostate include IL8, CCL2, CXCL12 and IL6 through the regulation of the transcription factors, NFκB, HIF1α and STAT3.[125] In addition, mutations and genetic polymorphisms of genes in inflammatory pathways have also been described.[126] The involvement of inflammation in PCa initiation and progression makes it a target in PCa prevention. Studies show that use of nonsteroidal anti-inflammatory drugs is associated with reduced PCa risk.[127-130]

Androgen-driven mechanisms

The normal development, growth and function of the prostate gland is dependent on androgens that exert their effects by binding to the androgen receptor (AR).[131] In the absence of androgen ligand, AR a nuclear receptor transcription factor, localizes to the cytoplasm where it is bound by heat shock proteins (HSP).[131] Upon androgen binding, the HSPs dissociate allowing AR to dimerize and translocate in to the nucleus where it mediates gene transcription.[131] Canonical global AR target genes are necessary for prostate growth and luminal differentiation.[132] Treatment with androgens promotes cell proliferation, survival and differentiation *in vitro*.[133,134] However, AR signaling also plays a significant role in PCa development and progression.

Recently, it has been suggested that switches in AR DNA binding activate non-canonical AR target genes overrepresented with genes involved in luminal epithelial de-differentiation and cell cycle genes enhancing tumorigenesis.[132] AR is also known to trigger the expression of the common PCa oncogenic gene fusionTMPRSS2-ERG.[131] The central role of AR in PCa makes it an attractive therapeutic target. Initially, the majority of prostate tumors are androgen dependent

and regress upon androgen deprivation with improvement of cancer related symptoms.[135] Inevitably however, the tumors recur and become castration resistant, with a median survival of 18-24 months.[131] The concurrent rise in serum PSA levels in CRPC indicates AR reactivation.[131]

Mechanisms mediating CRPC include; AR mutations that alter binding specificity, AR amplifications which increase androgen sensitivity, AR splice variants with a truncated ligand binding domain (LBD) allowing androgen-independent AR activation, coactivator and corepressor mutations and intratumoral androgen synthesis.[136] Efforts to find therapies for CRPC are ongoing.

Metabolic alterations in prostate cancer

Cellular metabolism and its reprogramming in cancer

Low levels of growth factors limit nutrient uptake in differentiated, quiescent cells which, depend on catabolic metabolism, mostly through oxidative phosphorylation (OXPHOS) to maximize ATP generation for normal cellular maintenance.[137] However, in the presence of abundant growth factors, proliferating cells couple catabolic metabolism to generate energy for replicative cell division with anabolic metabolism for biomass synthesis for the newly created cells and to maintain redox homeostasis.[137] By definition, cancer is uncontrolled cell proliferation and tumor metabolism parallels that of proliferating normal cells.[137] Altered metabolism to support rapid cell growth often in poorly vascularized, nutrient poor microenvironments is actively selected for in tumorigenesis, establishing it as a hallmark of cancer (Fig. 3).[137-139]

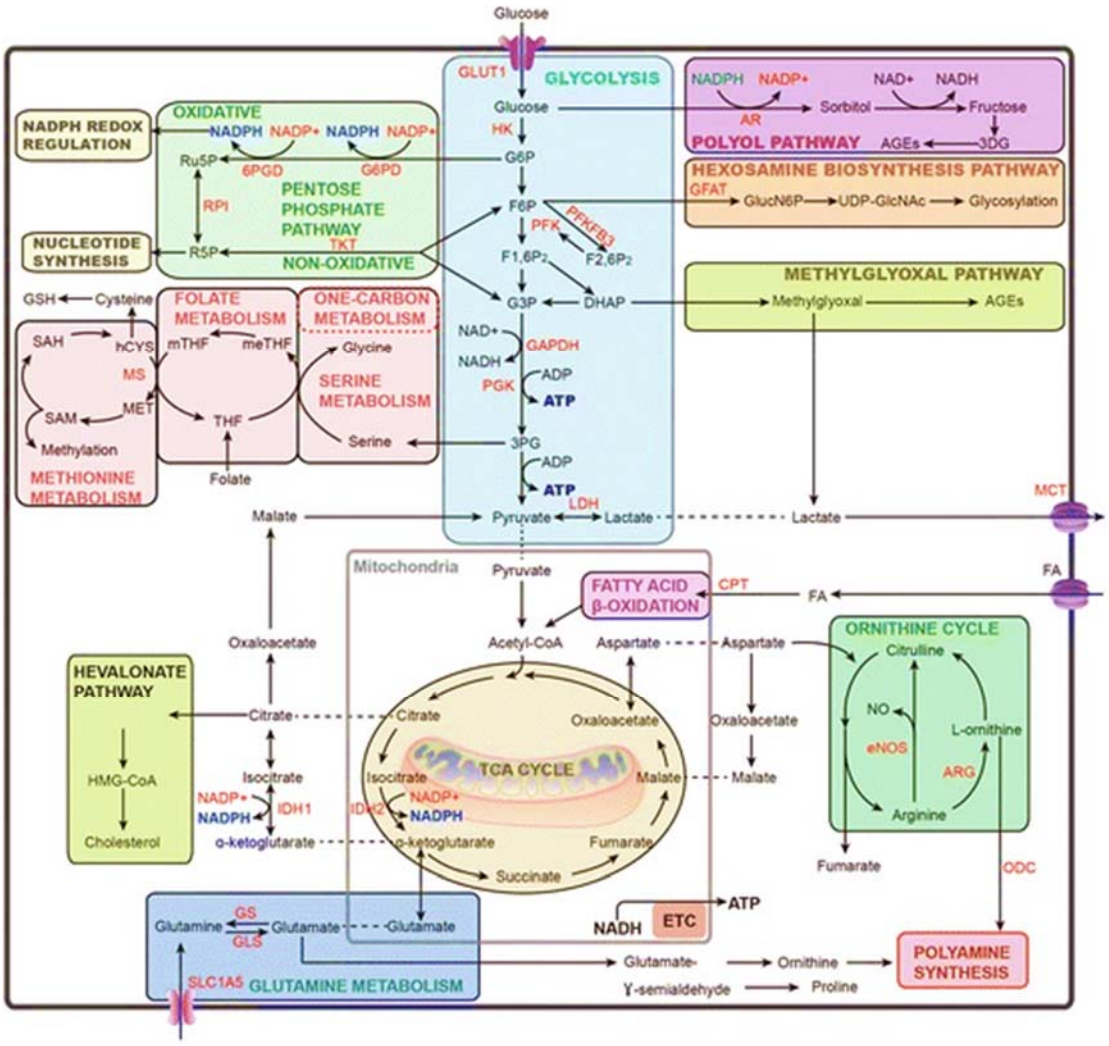

Figure 3. Main pathways in cancer metabolism. Explanations can be found in the text.

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Mammalian cells mainly depend on glucose, glutamine and lipids for biosynthesis and cell survival.[139] Glucose can be metabolized through glycolysis to pyruvate, which under anaerobic conditions is converted to lactate and two moles of ATP per mole of glucose in the cytoplasm.[140] In aerobic conditions, pyruvate is converted to Acetyl-CoA through oxidative decarboxylation by pyruvate dehydrogenase (PDH) in the mitochondria.[137] Oxidative phosphorylation (OXPHOS) of Acetyl-CoA in the mitochondria's TCA cycle produces the reducing equivalents NADH and FADH2, which mediate electron transfer in the electron transport chain (ETC) to generate the proton motive force that drives ATP synthesis.[139] OXPHOS yields 36 moles of ATP per mole of glucose.[140]

One of the earliest identified metabolic derangements in cancer cells is the increased glucose uptake for glycolysis even in normoxic conditions - the *Warburg effect*.[141] Proliferating cells are known to select for glycolysis because it generates intermediates for use in anabolic pathways.[142] For example, dihydroxyacetone phosphate (DHAP) is a precursor in the synthesis of structural cell membrane lipids triacylglyceride and phospholipids.[142] DHAP is also a precursor for cardiolipin a mitochondrial membrane lipid.[142] 3-phosphoglycerate is a precursor of sphingolipids a membrane component as well as the synthesis of the amino-acids serine, cysteine, and glycine.[142,143] Pyruvate can also be used to synthesize malate and alanine.[143]

Additionally, while glycolysis is less efficient for ATP generation relative to OXPHOS per mole of glucose, it allows for faster ATP generation in comparison.[141] Warburg's hypothesis that impaired mitochondrial respiration leads to the increase in glycolysis in cancer cells fueled the erroneous belief that cancer cells depend primarily on glycolysis for ATP production.[144] While 18F-fluorodeoxyglucose (18F-FDG) positron emission tomography validates that most human

tumors have increased glucose uptake compared to normal tissue, many tumors also concurrently retain oxidative metabolism.[143,145,146] Besides, diminishing ATP generation from glycolysis by inhibiting pyruvate kinase does not halt tumorigenesis.[147] Moreover, mitochondrial dysfunction impedes cell proliferation and tumorigenesis.[148-150]

Cancer cells therefore use OXPHOS for ATP generation in spite of high glycolytic flux unless their mitochondrial metabolic enzymes suffer disabling mutations.[151] Rather than damage to mitochondrial respiration, cancer cells have dysregulated glycolysis due to activation of oncogenes or loss of tumor suppressors.[141,151] In fact, rapidly proliferating cells like cancer cells can also obtain biosynthetic precursors from the TCA cycle for *de novo* macromolecule biosynthesis (cataplerosis).[152] Citrate can be used to generate acetyl-CoA and oxaloacetate.[153] Acetyl-CoA is a precursor for fatty acid synthesis.[153] Oxaloacetate can be converted to malate and then pyruvate generating NAD+ a glycolysis co-factor and NADPH a reducing equivalent.[153]

Oxaloacetate and α-Ketoglutarate can be converted into the amino acids aspartate and glutamate respectively, which then serve as precursors for purine synthesis.[153] Succinyl-CoA is important in the synthesis of porphyrin and heme, which are upregulated in breast and non-small-cell lung (NSCLC) cancers.[153] Besides ATP generation however, glucose has several other fates in the cell. Through one carbon metabolism, glucose is used for nucleotide metabolism, redox homeostasis, lipid biosynthesis, amino acid homeostasis and methylation metabolism.[154] Glucose can also be shunted through the Pentose Phosphate Pathway (PPP) to generate pentose phosphates, ribose-5-phosphates a precursor for ribonucleotides and NADPH a cofactor used in anabolic reactions.[155]

NADPH is a required electron donor for reductive steps in lipid synthesis, nucleotide metabolism and in maintaining GSH in its reduced state.[156] Glucose can also be metabolized through the hexamine biosynthetic pathway (HBP) to produce UDP-N-acetylglucosamine (UDP-N-GlcNAc).[157] UDP-N-GlcNAc is needed for *O*-linked posttranslational protein glycosylation and for the synthesis of glycosylphosphatidylinositol (GPI) anchors which secure proteins to membranes.[157] When TCA cycle precursors from glucose catabolism are withdrawn for biosynthesis, they are replenished using glutamine-derived α-ketoglutarate to maintain mitochondrial integrity- *anapleurosis*.[158]

Glutamine is also an important source of reduced nitrogen for the biosynthesis of purine and pyrimidine nucleotides, essential amino-acids, glutathione and lipid synthesis via reductive carboxylation.[159] In addition the conversion of glutamine-derived malate to pyruvate by malic enzyme is a source of NADPH.[160] Many cancers and proliferating cells metabolize more glutamine than is needed for protein and nucleotide synthesis, and some cancer cells depend on glutamine for survival in what is termed glutamine addiction.[161] In addition to glucose and glutamine, many cellular functions depend on lipids, and alterations in lipid and cholesterol metabolism are often seen in tumor cells.[162] Lipids are water insoluble molecules, comprising triacylglycerols, phosphoglycerides, sterols and sphingolipids.[163]

Since triacylglycerols are highly reduced and anhydrous, fatty acids provide twice as much ATP as carbohydrates or proteins and six times more ATP than glycogen, relative to their dry mass.[164] Fatty acids therefore preferentially serve as metabolic energy reservoirs under conditions of nutrient abundance in the form of lipid droplets (LD).[164] Lipids are critical components of biological membranes and cholesterol regulates membrane fluidity.[165,166] Cholesterol and

sphingolipids form membrane microdomains called lipid rafts, which concentrate receptors acting as hubs for signal transduction.[167] Cholesterol also participates in signaling as a precursor of steroid hormones including androgens, progestagens, glucocorticoids, mineralocorticoids, and estrogens.[168]

Additionally, intermediates of cholesterol biosynthesis, farnesyl pyrophosphate (FPP) or geranylgeranyl pyrophosphate (GGPP) activate Ras and Rho GTPases through posttranslational prenylation.[169] These small GTPases perform numerous cellular functions relevant to cancer, including control of cytoskeleton dynamics, migration, cell cycle progression, generation of ROS and gene expression.[170] Intermediates of *de novo* lipogenesis such as diacylglycerol (DAG) also serve as second messengers in cellular signal transduction.[166] Lipid mediated post translational modification regulates the localization and function of various signaling proteins like GPI (glycosylphosphatidylinositol) anchored proteins some of which are associated with cancer, like the urokinase-type plasminogen activator (uPA)-receptor (uPAR).[166]

Protein S-palmitoylation, involving the formation of a thioester bond between a cysteine thiol side chain with the 16-carbon fatty acid palmitate, is a widespread lipid modification crucial for regulating protein subcellular localization, stability among other functions.[166] The oncogenic activation of β-catenin through Wnt palmitoylation, which is mediated by fatty acid synthase (FASN) might play a role in PCa.[166] Protein N-myristoylation a lipid modification by N-myristoyltransferase (NMT) that attaches myristate, a 14-carbon fatty acid, to protein N-terminal glycine residues regulates cellular localization and signal transduction.[171] Myristoylation activates the serine threonine kinase Akt leading to neoplastic transformation.[166]

Cancer cells display a great demand for lipids and cholesterol. With the exception of the liver, adipose tissue and mammary cells which synthesize fats *de novo*, most adult normal cells preferentially use circulating fatty acids (FAs) or those complexed with proteins *e.g.* low-density lipoproteins from dietary sources or from lipogenic tissues.[165] To provide lipids for membrane production, a lot of cancers synthesize lipids and cholesterol *de novo* and to a lesser extent increase the uptake of exogenous fats; LD and cholesterol levels correlate with tumor aggressiveness.[162,165] Lipid synthesis (lipogenesis) starts with the conversion of citrate from the TCA cycle in to acetyl-CoA and oxaloacetate by ATP-citrate lyase.[165] Glucose and glutamine can both contribute Acetyl-CoA for fatty acid synthesis.[172]

Pyruvate dehydrogenase (PDH) converts glycolysis-derived pyruvate to Acetyl-CoA.[172] Glutamine-derived α-ketoglutarate is reductively carboxylated to isocitrate (by isocitrate dehydrogenase 1, IDH1) then citrate (by aconitase) which is cleaved to oxaloacetate and Acetyl-CoA.[172] Acetyl-CoA is converted to Malonyl-CoA by acetyl-CoA carboxylase (ACC) which is rate limiting in lipid synthesis.[166] Subsequently, fatty-acid synthase enzyme (FASN) synthesizes the saturated fatty acid palmitate (16:0) with Acetyl-CoA as a primer, Malonyl-CoA as a two-carbon donor and NADPH the reducing equivalent.[166] To generate NADPH, oxaloacetate can be converted to malate then pyruvate by malate dehydrogenase and malic enzyme respectively.[166] Alternatively, NADPH could be derived from the PPP pathway.

Saturated FAs are elongated or desaturated by elongase and desaturase enzymes (like Stearoyl-CoA desaturase, SCD1), respectively, to generate various long chain saturated or desaturated fatty acids.[165] Cholesterol on the other hand is synthesized from Acetyl-CoA in the mevalonate pathway.[165] The process begins with the condensation of three Acetyl-CoA molecules by

acetoacetyl-CoA thiolase and HMG-CoA synthase to form 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA).[173] In the subsequent rate-limiting step, HMG-CoA is reduced to mevalonate in the presence of NADPH by HMG-CoA reductase (HMGCR) an enzyme under complex regulatory control.[173] When sterol isoprenoids are in short supply, sterol regulatory element binding proteins (SREBP) bind to sterol regulatory elements (SREs) on the HMGCR promoter increasing its transcription.[173]

When sterol levels are sufficient, the HMGCR sterol-sensing domain mediates its ubiquitin mediated degradation.[173] During metabolic stress however, AMP-activated protein kinase (AMPK) phosphorylates and deactivates HMGCR lowering sterol metabolism.[173] Mevalonate is then converted to isoprenoids including cholesterol by various enzymes in the melavonate pathway.[165] During starvation and in certain situations like loss of matrix attachment however, cancer cells activate fatty acid β-oxidation (FAO) to generate acetyl-coA and ATP.[174,175] While short and medium chain fatty acids are able to diffuse in to the mitochondria for FAO, the mitochondrial membranes are impermeable to long chain fatty acids.[176]

Prior to transportation in to the mitochondria, long chain fatty acids are first be activated in to fatty acyl-CoA by acyl-CoA synthetase (ACS) in the cytosol.[176] In FAO's rate limiting step, carnitine palmitoyltransferase-1 (CPT1) on the outer mitochondrial membrane conjugates fatty acyl-CoAs to carnitine forming acylcarnitines for transportation into the mitochondrial inter membrane space (IMS).[177] Transport from the IMS to the mitochondrial matrix is facilitated by carnitine–acyl carnitine translocase (CACT).[178] CPT2 located on matrix side of the inner mitochondrial membrane regenerates fatty acyl-CoA from the fatty acyl carnitine for β-oxidation and the free carnitine is transported back in to the cytosol by CACT.[176]

In FAO, repeated cycles of oxidation, hydration, another oxidation and thiolytic cleavage shorten even-chain-length fatty acyl-CoA molecules two carbons per cycle to generate acetyl CoA, NADH and FADH2.[175] The shortened fatty acyl-CoA renters the β-oxidation cycle until a four-carbon fatty acyl molecule is reduced to two acetyl-CoA molecules that are fed in to the TCA cycle and the electron transport chain for ATP synthesis.[175] Odd-chain-length fatty acyl-CoA molecules, a rare species, also undergo the same oxidation pathway but they yield both actely-CoA and propionyl-CoA as end products.[179]

The last β-oxidation cycle of odd-chain-length fatty acyl-CoAs cleaves five carbon acyl-CoA molecules in to a three carbon molecule (propionyl-CoA) and a two carbon molecule (Acetyl-CoA).[179] The propionyl-CoA is converted into succinyl-CoA before entry in to the TCA cycle.[179]

Mechanisms of metabolic reprogramming in cancer cells

Metabolic reprogramming in cancer cells is mainly driven by oncogenes, loss of tumor suppressors and to a lesser extent, mutations in metabolic enzymes.[151] MYC, an oncogene often upregulated or amplified in cancer, transcriptionally upregulates nutrient transporters, glycolytic and glutaminolytic enzymes as well as mediating glutamine addiction in some cancers.[180] The tumor suppressor p53 induces TIGAR (TP53-induced glycolysis regulator) that converts fructose-2,6-bisphosphate an allosteric activator of the glycolysis rate limiting enzyme PFK1 to fructose-6-bisphosphate downregulating glycolysis and increasing PPP flux.[155] Loss of p53 as seen in half of human cancers therefore results in increased glycolytic flux and ROS accumulation that promotes tumorigenesis.[151]

The PI3K/AKT/mTOR pathway, which is abnormally activated in many cancers, increases glycolysis and *de novo* lipogenesis via the hypoxia-inducible factor (HIF1) and SREBP respectively.[181-183] BRAF and KRAS oncogenes also enhance glycolysis by activating AKT.[155] HIF1 increases GLUT1 and hexokinase 2 (HK2) expression upregulating glucose uptake and glycolysis respectively.[184,185] Besides the hypoxic conditions found in most tumors activate HIF1 increasing glycolysis.[151]

However, some tumors can constitutively activate HIF1 even under normoxic conditions through several mechanisms, including loss of von Hippel-Lindau (pVHL) which targets hydroxylated HIF for ubiquitin-mediated degradation, accumulation of ROS and metabolic signaling.[151] Inactivating mutations in succinate dehydrogenase (SDH) and fumarate hydratase (FH) lead to mitochondrial accumulation of their substrates succinate and fumarate, respectively, which inhibit HIF prolyl-4-hydroxylases (PHDs) when they leak into the cytosol preventing HIF1 degradation.[186] Other mutational mechanisms leading to metabolic reprogramming have also been described.

Phosphoglycerate dehydrogenase (PHDGH) diverts 3-phosphogycerate, a glycolytic metabolite, into the serine biosynthetic pathway, which contributes one-carbon units for methylation reactions, nucleotide and NADPH biosynthesis.[151] The PHDGH gene is amplified in approximately 6% of breast cancers and 40% of melanomas, and tumors that overexpress it are dependent on it for growth.[187] In gliomas and acute myeloid leukemia (AML), recurrent mutations lead isocitrate dehydrogenase 1 and 2 (IDH1/2) to lose their normal ability to interconvert isocitrate to α-ketoglutarate.[137] Instead, mutated IDH1/2 reduce α-ketoglutarate to D-2-hydroxyglutarate (D2HG) a rare metabolite.[137]

Increased concentration of D2HG in tumors inhibits several α-ketoglutarate-dependent dioxygenase enzymes including the TET family of enzymes which methylate DNA altering gene expression.[137] Besides mutations, tumors preferentially express specific isoforms of metabolic enzymes.[137] Cancer cells preferentially express the M2 isoform of the glycolytic enzyme pyruvate kinase (PKM2) that favors anabolic metabolism over the M1 isoform that is predominant in differentiated, quiescent tissue.[137]

Metabolic cancer therapies

Though metabolic reprogramming contributes to tumorigenesis, it also offers metabolic vulnerabilities that can be exploited for cancer therapy.[188] However, normal proliferating cells in bone marrow, intestinal epithelium and hair follicles also reprogram their metabolism in a manner similar to tumor cells reducing the therapeutic window for anti-proliferative metabolic therapies.[188] The feasibility of anti-metabolic therapies depends on how well they can be tolerated because disrupting the function of metabolic enzymes in normal tissue can result in systemic toxicity.[151] However, for decades, antifolates (like methotrexate, aminopterin and pemetrexed), which target nucleotide generating one-carbon metabolism, have been crucial components in chemotherapies.[188]

The clinical success of antifolates for cancer therapy is predicated on the increased demand for nucleotide synthesis and DNA replication in neoplastic cells.[188] Although antifolates do cause toxicity in rapidly proliferating tissue, they are integral in many chemotherapy regimens that increase patient survival.[188] As amino-acids drive protein synthesis, mitochondria metabolism and form intermediate metabolites for biosynthetic pathways, several therapies target amino-acid

metabolism.[140] The FDA has approved L-asparaginase which depletes plasma asparagine as an anti-cancer treatment in patients with acute lymphoblastic leukemia (ALL).[140]

Whereas asparagine synthetase (ASNS) can synthesize asparagine *de novo* in normal cells, low ASNS expression sensitizes leukemic lymphoblasts to asparagine withdrawal, which diminishes asparagine-dependent protein synthesis and induces cell death.[189] Also, given that cancer cells upregulate lipid and cholesterol synthesis for membrane synthesis and for lipid signaling intermediates, inhibiting enzymes in these pathways could be an effective anti-cancer strategy. Several clinical trials are evaluating the efficacy of statins, which inhibit cholesterol synthesis through HMGCR and are approved for treating cardiovascular disease, as anti-cancer therapy.[140]

Mitochondrial metabolism is another emerging therapeutic target because it is frequently upregulated in cancer to meet increased demand for ATP and biosynthetic precursors.[140] Metformin, an antigluconeogenic drug approved to treat type 2 diabetes also inhibits mitochondrial complex I in cancer cells decreasing ATP generation and inducing apoptosis in glucose limiting conditions.[151] Epidemiologic data also suggest that metformin lowers the risk of cancer development in diabetic patients who are cancer naïve and increases survival in diabetic patients with cancer.[151] Moreover, the selective uptake of metformin by organic cation transporters (OCTs) expressed in some tumors and in a few tissues like the liver makes it well tolerable.[151]

Metformin's efficacy against cancer is currently being assessed in numerous clinical trials in non-diabetic patients.[140] Additionally, the FDA has approved mitochondrial complex III inhibiting arsenic trioxide for the treatment of relapsed/refractory acute promyelocytic leukemia

(APL) and it is also being tested on other cancers.[140] Another potential therapeutic strategy against mitochondrial metabolism would be the inhibition of mitochondrial enzymes.[140] There are ongoing trials to test inhibitors of mutated IDH1/2 in glioma and AML patients which convert α-ketoglutarate to the oncometabolite D2HG, a unique feature of cancer metabolism.[140] By inhibiting pyruvate dehydrogenase kinase (PDK), dichloroacetate (DCA) increases the conversion of Acetyl-CoA from pyruvate by pyruvate dehydrogenase (PDH) decreasing glycolytic flux, and inducing apoptosis in cancer cells with deficient mitochondrial function.[140]

Several clinical trials have evaluated DCA in cancer patients though its clinical efficacy may depend on a tumor's metabolic profile.[140,190] As mitochondrial metabolism is linked to oxidative stress, focal and metastatic cancer cells increase their antioxidant capacity to counter ROS production.[151] Alternative therapeutic strategies target redox metabolism by inhibiting antioxidants; the resulting accumulation in ROS causes apoptosis.[151] MTHFD2 an enzyme in the folate pathway that generates NADPH needed to maintain several antioxidant systems is overexpressed in 19 different cancers but not in normal cells making it a potential therapeutic target.[191]

In vitro, suppression of MTHFD2 in cancer cells alters multiple metabolic pathways and increases sensitivity to oxidant-induced apoptosis.[192-194] In cells, the oxidized form of Vitamin C, dehydroascorbate (DHA), preferentially transported by the glucose transporter GLUT1 which is often upregulated in tumors, is reduced to vitamin C using glutathione (GSH), and NADPH.[195] High doses of Vitamin C administered to mice harboring KRAS and BRAF mutant colorectal cancers is oxidized to DHA in the bloodstream and its uptake depletes NADPH and GSH, leads to metabolic collapse and induces cell death.[196] As next generation sequencing enables tumor

molecular profiling there is interest in personalized cancer therapy where drugs are tailored against driver mutations in individual patients as opposed to depending solely on clinical factors.[197]

These efforts are however hampered by intertumoral and intratumoral genomic heterogeneity.[197] Some argue that despite diverse mutations in tumor suppressors and oncogenes, they drive common metabolic phenotypes and so metabolism might be a better target for cancer therapy.[140] However plastic metabolic phenotypes exist within and among tumors under influence from the tumor microenvironment.[198] Understanding the metabolic reciprocity among tumor cells and between tumor and stromal cells will help in the development of effective metabolic therapies.[151]

Metabolic reprogramming in prostate cancer

Prostate secretory epithelial cells in the peripheral zone are metabolically adapted for the specialized function of producing prostatic fluid.[199] In the most mammalian cells, glycolysis-derived pyruvate is decarboxylated to Acetyl-CoA, which is condensed with oxaloacetate forming citrate which is oxidized for ATP generation in the TCA cycle.[200] In contrast, high expression of ZIP1 and ZIP3 zinc transporters in secretory prostate epithelial cells leads to high zinc accumulation, which inhibits mitochondrial aconitase (m-aconitase) blocking citrate oxidation.[201-205] Attenuation of the TCA cycle and additional synthesis of citrate by prostate cells lead to net citrate accumulation in the prostatic fluid for subsequent secretion into the seminal fluid.[204]

Seminal fluid contains up to 12-fold higher levels of citrate compared to blood plasma.[204] Citrate is believed to act as a buffering agent and a chelator of calcium and zinc.[206-208] The use of

glycolysis-derived pyruvate to generate Acetyl CoA for citrate synthesis in the prostate is impeded by the slow rate of glycolysis in normal prostate cells.[209] In fact, slow glucose uptake by prostate cells attributed to low expression of glucose transporter 1 (GLUT1), has precluded the use of FDG-PET for the detection and staging of PCa.[210] Diminished glycolysis in the normal prostate has also been linked to the inhibitory effect of high levels of citrate on phosphofructokinase a key glycolytic enzyme.[209]

Unlike most other solid tumors, prostate tumors only exhibit a glycolytic phenotype in metastatic or castration resistant stages as evidenced by the accumulation of 18F-FDG and the expression of glycolytic markers and monocarboxylate transporters.[211,212] In the face of limited glycolysis, FAO has been proposed to be the major source of Acetyl-CoA for prostate citrate production.[213] Benign prostate cells preferentially use fatty acids over glucose to meet their bioenergetics needs, and they also contain a higher than usual intracellular cholesterol content.[213-215] In the prostate, oxaloacetate for citrate production is derived from the transamination of aspartate by mitochondrial aspartate aminotransferase (mAAT).[199] The prostate epithelium reportedly uses the excitatory amino acid carrier 1 (EAAC1) as a high affinity aspartate transporter.[216]

The transformation of prostate epithelial is accompanied by the loss of zinc accumulation due to decreased expression of zinc transporters.[217] Downregulation of Zinc transporters in PCa cells occurs partly through epigenetic silencing of the ZIP1 and ZIP3 transcription regulator, AP-2alpha, through promoter hypermethylation.[218] DNA demethylation using 5-aza-2'-deoxycytidine increased the expression of ZIP1 and ZIP3 and Zinc uptake in DU-145 and LNCaP PCa cell lines.[218] Loss of zinc accumulation also inhibits its non-metabolic anti-tumorigeneic effects aiding prostate tumorigenesis. For example, supplementation of PCa cell lines with physiological

amounts of zinc induces apoptosis by stimulating Cytochrome C release and suppression of the antiapoptotic protein c-IAP2 downstream of NF-κB.[219,220]

Zinc supplementation also reduces invasiveness by downregulating ICAM-1 (Intercellular Adhesion Molecule 1) and inhibits NF-κB pro-angiogenic and pro-metastatic effectors.[221] More importantly however, low zinc reactivates m-aconitase leading to citrate oxidation to meet the cells' growing energetic needs.[199,222,223] The switch to citrate oxidation is thought to be an early event in the progression of PCa preceding detectable histological changes.[223-225] However, citrate oxidation is necessary but insufficient for full transformation.[200] PCa cells need additional metabolites for anabolic and energy needs some of which they obtain from cells in the tumor microenvironment.[200] Stromal cells and prostate epithelial cells have been shown to mutually reprogram each other's metabolism to enhance tumorigenesis.[226,227]

Fiaschi *et al.*, reported that physical contact with PCa cells activates stromal fibroblasts triggering a glycolytic phenotype with increased GLUT-1 expression, glucose metabolism and oxidative stress.[226] The activated fibroblasts increased lactate production and efflux through newly expressed monocarboxylate transporter-4 (MCT4).[226] Conversely, fibroblast contact increased OXPHOS and lactate uptake via the MCT1 lactate transporter but downregulated GLUT-1 expression in PCa cells.[226] Eventually, the PCa cells ceased metabolizing glucose relying instead on lactate demonstrating how PCa cells could use cancer associated fibroblasts (CAFs) to adapt to low glucose environments.[226] In this case, the reciprocal metabolic programming between stromal and PCa epithelial cells was regulated by HIF-1, which was stabilized by Sirtuin-3 even under normoxic conditions.[226]

Valencia *et al.*, showed that a decrease in stromal p62 downregulates mTORC1 and MYC reducing GSH and NADPH synthesis from glucose and glutamine.[227] Diminished redox metabolism in p62 deficient prostate stromal fibroblasts led to oxidative stress which stimulated IL6 secretion triggering TGFβ synthesis.[227] TGFβ activated the fibroblasts, which enhanced prostate tumor growth and invasion *in vivo*.[227] CAFs also mediate post-translational modifications important for pyruvate kinase M2 (PKM2) nuclear localization where it complexes with HIF1 and the transcriptional repressor DEC1 (Differentially Expressed in Chondrocytes-1).[226,228] The trimeric complex downregulated miR205 expression, which switched the metabolism of PCa cells to OXPHOS during PCa progression.[228]

PCa also displays increased glutamine metabolism in the more aggressive metastatic stages primarily for TCA cycle anaplerosis.[229] Glutaminase (GLS) converts glutamine to glutamate, a source of the TCA cycle intermediate α-ketoglutarate (α-KG).[230] PCa cells overexpress GLS1 and in patients GLS1 expression correlates highly with PCa tumor stage and disease progression.[231] In PCa cells, MYC promotes glutaminolysis by upregulating the expression of GLS1 through repression of miRNA-23a/b.[232] Withdrawing glutamine or knocking down GLS in glutamine addicted PC3 PCa cells decreased ATP, cell proliferation and GSH levels causing ROS-induced apoptosis.[232,233] In addition to glutaminolysis, GLS1 downregulates the glycolytic repressor, thioredoxin interacting protein (TXNIP) increasing glucose utilization in PCa cells.[231]

Coordination of glutamine and glucose metabolism is thought to constitute a metabolic checkpoint that restricts cell growth when levels of either nutrient is limiting.[231] Glutamine is imported in to cells by the solute carrier (SLC) group of transporters.[230] PCa cells overexpress glutamine transporters ASCT2 (or SLC1A5) and SLC1A4 due to multiple oncogenic

signals.[234,235] AR mediated signaling drives glutamine dependent proliferation of prostate cells by upregulating the expression of SLC1A4 and SLC1A5 glutamine transporters through mTORC1.[234] MYC also regulates AR mediated glutamine uptake but only in PTEN null prostate cells.[234] Additionally, the constitutively active androgen-receptor splice variant 7 (AR-V7) expressed in CRPC increases dependence on glutaminolysis for TCA cycle anaplerosis as well as glutamine reductive carboxylation.[236]

Inhibition of ASCT2 in prostate cells decreased glutamine uptake and fatty acid synthesis reducing the growth of cancer cells and tumor xenografts as well as metastasis.[235] On a molecular level, ASCT2 inhibition downregulated E2F cell cycle proteins and mTORC1 activation.[235] Glutamine metabolism in PCa cells can also be affected by exosomes secreted by cancer-associated fibroblasts (CAFs).[237] Addition of CAF-derived exosomes (CDEs) to PCa cells increased ready-made metabolites, decreased OXPHOS and increased glycolysis as well as reductive carboxylation of glutamine.[237] These findings further demonstrate how tumors might use stromal CDEs to reprogram their metabolism in nutrient deficient conditions.[237]

An aberrant increase in *de novo* lipogenesis is another common occurrence in early clinical PCa.[166] The peripheral zone which shows the highest propensity for PCa development has increased transcription of lipid biosynthesis enzymes with a concomitant rise in fatty acid metabolites.[238] Paradoxically, this zone also displays increased fatty acid catabolism indicating an increased capacity for lipid metabolism in general.[238] Overexpression of the lipogenic enzyme FASN occurs early in PCa tumorigenesis with both mRNA and protein being detected in increasing amounts from PIN to metastases.[239-241] FASN overexpression is associated with PCa progression and it has been linked to poor prognosis and reduced disease free survival.[242,243] In

addition, nuclear localization of FASN in a subset of PCa cells has been associated with a more aggressive phenotype.[244]

Moreover, overexpression of FASN transforms immortalized, benign prostate epithelial cells and FASN transgenic mice develop PIN.[245] FASN oncogenesis in PCa has been linked to Wnt1 palmitoylation which results in cytoplasmic accumulation of β-catenin, inhibition of apoptosis, protection from ER stress and production of lipids for membrane synthesis.[245-247] In addition, relative to matching normal tissue, FASN expressing prostate tumors have elevated levels of saturated and mono-unsaturated fatty acids and decreased polyunsaturated fatty acids.[248] The switch to more saturated fatty acid species protects cells from oxidative stress and doxorubicin-induced cell death.[248] In addition, as saturated phospholipids are preferentially incorporated in to lipid fats, increasing their synthesis would significantly impact the lipid raft signaling landscape.[249]

Inhibition of FASN decreased cell viability, proliferation, migration and clonogenic survival while increasing the selective apoptosis of different human cancer cells including prostate.[245,250-253] *In vivo*, FASN inhibition reduced the growth of LNCaP tumors pointing to the importance of a lipogenic phenotype for PCa growth and survival.[254] FASN overexpression in the prostate has been linked to several mechanisms. FASN copy number alterations have been detected in some PCa cells lines as well as adenocarcinoma and metastatic cancers implicating gene amplification in increased FASN expression.[255] Increased H3K27Ac marks in the FASN gene promoter by P300 upregulated FASN expression leading to lipid accumulation *in vitro* and in the prostates of mice with a PTEN knockout.[256]

Growth factors and their receptors as well as hormones can also upregulate FASN expression. In prostate cells, androgens and EGF can stimulate SREBP-1 which in turn regulates the expression of AR in a feedback loop that further drives the expression of lipogenic enzymes.[257-259] In PTEN null, LNCaP as well as human prostate tissues, FASN expression was linked to the activation of the PI3K/Akt pathway.[260-262] Accordingly, inhibition of PI3K/AKT in PCa cells reduces FASN expression.[262,263] Post-translational modifications that stabilize FASN in prostate cells have also been described.[264] The deubiquitinating enzyme USP2a prevents FASN proteasomal degradation with its knockdown decreasing levels of FASN.[264]

Besides FASN, inhibition of ACYL, another lipid synthesis enzyme, reduces PCa cell viability in the absence of exogenous lipid supplies.[265] Concomitant inhibition of ACYL and AR in CRPC cells induced energetic stress, activated AMPK and synergistically suppressed AR decreasing cell proliferation and increasing apoptosis.[266] There are conflicting reports on the expression of SCD1 in PCa. One study reported frequent loss of SCD1 transcripts and protein in human PCa tumors compared to benign tissue.[267] Different studies report that SCD1 is upregulated in PCa increasing the ratio of monounsaturated to saturated fatty acids.[268] High intracellular accumulation of saturated fatty acids has been linked to lipotoxicity so SCD1 overexpression in cancers might protect cells from such toxicity.[166,269]

Overexpression of SCD1 increased tumorigenecity in androgen sensitive LNCaP cells and tumor growth in nude mice.[270] Inhibiting SCD1 decreased lipid synthesis, cell proliferation and *in vivo* tumor growth in nude mice and prostate orthografts while increasing survival.[268,271] SCD1 inhibition downregulated the AKT pathway possibly through decreased levels of PIP3, (phosphatidylinositol-3,4,5-trisphosphate (PI (3,4,5) P3)).[268] In addition, SCD1 inhibition

activated AMPK and decreased β-catenin, which activated glycogen synthase α/β (GSK3 α/β).[268] Furthermore, SCD1 activity appears to be important in Ras oncogene cell transformation.[268] SCD1 has also been implicated in AR activation in LNCaP cells.[270]

High-grade PCas also overexpress the fatty acid elongation enzyme ELOVL7 through the activation of SREBP1 by the androgen pathway.[272] Knockdown of ELOVL7 altered phospholipids and neutral lipids like cholesterol a precursor for androgen synthesis.[272] Consequently, knockdown of ELOVL7 decreased androgen synthesis and attenuated PCa cell proliferation.[272] Consumption of a high fatty diet on the other hand increased tumor growth in ELOVL7 expressing tumors linking diet and PCa.[272] As NADPH and acetyl-CoA from glucose and glutamine metabolism can drive lipid biosynthesis, increased lipogenesis in cancer cells could result from the upregulation of these two pathways and not necessarily the dysregulation of lipid synthesis enzymes.[166]

In advanced PCa, upregulation of SREBP2 that transcriptionally regulates enzymes involved in cholesterol biosynthesis and loss of sterol feedback inhibition increases androgen synthesis.[273,274] In PCa cells, promoter hypermethylation of the cholesterol efflux protein ABCA1 downregulates its expression altering cholesterol homeostasis.[275] ABCA1 downregulation correlates inversely with Gleason grade implicating high cholesterol levels to cancer progression.[275] Besides promoting androgen synthesis, cholesterol in lipid rafts also promotes PCa progression by activating AKT signaling.[276,277] Metabolic intermediates in cholesterol synthesis are used to prenylate small GTPases like RhoC GTPase, increasing proliferation and metastasis in PCa.[278]

Additionally, major structural components of the cholesterol-enriched invaginations of the plasma membrane caveolae called caveolins (CAV) are upregulated in PCa, and they are linked to disease progression.[279,280] CAV-1 is suggested to have a role in the modulation of cholesterol in the mitochondria as well as mediating androgen receptor activity.[281,282] Deletion of Cav-1 decreased tumor burden in the prostates of TRAMP mice as well as lymph node metastases.[283] Epidemiologic data indicates that the use of statins (HMG-CoA reductase inhibitors) is associated with a lower risk of developing advanced PCa.[284] Statins have therefore been proposed for secondary and tertiary PCa chemoprevention.[284]

Dysregulation in fatty acid catabolism has also been demonstrated in human cancers, including PCa. AMACR (alpha-methylacyl-CoAracemase), which controls β-oxidation of dietary branched chain fatty acids in peroxisomes is frequently upregulated in PIN and PCa and its expression is associated with an increased risk of disease.[285-287] In addition, treatment of PCa cells *in vitro* with branched fatty acids from dairy and beef products increased the expression AMACR.[288] Increased expression of pristanoyl-CoA oxidase (ACOX3), which is downstream of AMACR, further points to the importance of perosisomal β-oxidation of branched-chain fatty acids in PCa.[289] Perosisomal β-oxidation has been linked to the production of reactive oxygen species (ROS), which might contribute to carcinogenesis through DNA damage.[290]

Oxidative stress driven mechanisms

Reactive oxygen species (ROS) are reactive molecules derived from the partial reduction of molecular oxygen (0_2). The source of intracellular ROS can be exogenous or endogenous from normal cellular processes.[291] Endogenous ROS are by-products of aerobic respiration and protein

folding or produced by NADPH oxidase (NOX) complexes and inflammation.[291] ROS include free radicals such as the oxygen radical ($O_2^{\cdot\cdot}$), hydroxyl ($OH^{\cdot}$), superoxide anion ($O_2^{-\cdot}$) and peroxide radicals ($ROO^{\cdot}$) but also non-radicals like hydrogen peroxide (H_2O_2) and singlet molecular oxygen ($^1O_2$).[291] Non-radicals can give rise to free radicals for example in the presence of transition metals.[291]

In small quantities ROS take part in signal transduction (*i.e.* redox signaling) by reversibly oxidizing protein thiol groups affecting numerous physiological processes including hypoxia, gene transcription through redox sensitive transcription factors, cell proliferation and differentiation.[292,293] However, high levels of ROS result in the indiscriminate damage of cellular macromolecules; lipids, proteins, and DNA.[294] Oxidative DNA damage is thought to play a critical role in all stages of carcinogenesis.[295] ROS can modify DNA bases, form DNA adducts, induce DNA cross-linking and cause DNA strand breaks.[296] Oxidative DNA lesions that are not removed prior to DNA replication can lead to replication errors, mutations and genome instability, increasing the risk of carcinogenesis.[297]

ROS also indirectly increase cancer risk if they cause lesions on tumor suppressor genes or inactivate negative regulators of oncogenes and DNA repair proteins.[298,299] Under physiological conditions, ROS accumulation is regulated by endogenous enzymatic and non-enzymatic antioxidant defense systems.[291] Antioxidant enzymes include glutathione peroxidase (GPX), Gltathione S-transferase (GST), catalase (CAT) and superoxide dismutase (SOD).[291] Non-enzymatic antioxidants include vitamin C and E, flavonoids and low molecular weight co-factors and peptides like glutathione (GSH), NADPH and peroxiredoxin (PRX).[291] Oxidative stress

occurs when ROS levels outstrip these cellular antioxidant defenses.[300] Epidemiological, experimental and clinical data link oxidative stress to PCa development and progression.

In Nkx3.1 prostate specific knockout (KO) mice, dysregulation in the expression of antioxidant and prooxidant enzymes led to oxidative stress and development of HGPIN while Nkx3.1/Pten double heterozygous mice sustained more oxidative damage and progressed to adenocarcinoma.[301] In the transgenic adenocarcinoma of the mouse prostate (TRAMP) model of PCa, products of oxidative damage; 8-hydroxy-2'-deoxyguanosine (8-OHdG), 4-hydroxynonenal (4-HNE)-protein-adducts and nitrotyrosine (Ntyr), could be detected in early prostatic tumorigenesis.[302] Administration of testosterone and β-estradiol triggered the expression of prooxidant enzymes and oxidative damage, which induced stromal inflammation and dysplasia in the lateral prostate (LP) of the noble (Nb) rat hormonal carcinogenesis model.[303] These studies implicate oxidative stress from genetic perturbations or sex hormones in PCa initiation and progression.

Relative to benign prostate cells, human PCa cell lines have higher oxidative stress which is associated with a more aggressive phenotype.[304] Besides, transcription factors important for PCa like NK-κB, AP-1, HIF-1 and p53 are redox sensitive.[305] Several PCa risk factors, including age, diet, inflammation and androgens, are also associated with oxidative stress.[306] Moreover, key genetic and epigenetic changes in PCa have been shown to decrease the expression of genes important for prostatic redox homeostasis like GSTP1, NRF2, NKX3.1 and NADPH oxidases.[301,304,307,308] Accordingly, PCa patients show increasing oxidative biomarkers, including thiobarbituric acid reactive substances (TBARS), 8-OHdG and 4HNE-modified proteins, concomitant with loss of antioxidant defenses with disease progression.[309-314] These data are the

basis of the belief that antioxidants, by inhibiting oxidative stress, could be chemopreventive against PCa.

Prostate cancer diagnosis and treatment

Screening

In the U.S., the prostate-specific antigen (PSA) is used for PCa screening and early detection followed by a confirmatory biopsy.[315] Digital rectal examination (DRE) was used prior to PSA testing, but its use for primary PCa screening is limited by poor sensitivity and specificity and high inter-observer variability.[316,317] A normal PSA has a common arbitrarily threshold of ≤ 4.0 ng/mL, but this only has a 30% positive predictive value in men ≥ 50 years and 85% negative predictive value in men with a median age of 69 years at biopsy.[318] Widespread PSA screening in the U.S. shifted the diagnosis stage away from metastases in the 1980s and '90s and increased detection of curable early-stage cancers.[319]

PCa specific mortality also decreased by 4% by 1992 (5 years after the introduction of PSA screening) though it remains contentious whether the decrease is directly attributable to PSA testing.[320-324] Besides, PSA is not a PCa specific marker. Serum PSA can increase due to disruption of normal prostate anatomy by other prostatic diseases, including benign prostatic hyperplasia (BPH), prostatitis and prostatic trauma.[325,326] PSA also has low specificity with 75% men with a PSA > 3ng/mL having a negative biopsy while 7 – 56% of those with a positive biopsy would have died without clinical disease and therefore considered "overdetected".[327,328]

Lack of PSA specificity leads to unnecessary biopsies which carry the risk of bleeding and infection, detection and overtreatment of clinically insignificant PCa with accompanying treatment related morbidities.[329] On the flip side, high-grade cancers can cause deceptively low serum PSA levels that would be missed under the PSA guidelines for a biopsy.[330] The inability to distinguish between indolent and lethal PCas remains a major clinical challenge. Assessing prostate morphology combined with function and physiology using multiparametric magnetic resonance imaging (mpMRI) might decrease the risks associated with PCa screening and treatment.[331,332]

Biopsy

The need for a prostate biopsy is determined by PSA levels, a suspicious DRE, patient's age, and comorbidities.[333] Multifocality in PCa is common so to increase cancer detection rates, (CDRs), the American Urological Association (AUA) recommends surveying 10–12 cores of prostate tissue including far lateral and apical samples, in the initial biopsy.[334] Pathological evaluation is based on the Gleason grade, which is a five-tier grade system representing a continuum of histological and differentiation of the prostate.[335] The Gleason score (ranging from 2 to 10) comprises the most prevalent pattern or the primary grade and the second most prevalent pattern, the secondary grade.[335] For biopsies with cancer, Gleason score is a major determinant of treatment options as well as the tumor volume, which is estimated from the number of cores with cancer.[335]

Treatment

The therapeutic management of PCa depends on a patient's age and life expectancy, presence of comorbidities, patient's preferences and the probability of recurrence and metastasis after treatment (risk stratification).[336] Depending on PSA level, Gleason score, tumor grade and the degree of prostate involvement (number of biopsy cores with cancer) patients are divided into several prognostic groups as very low-risk, low risk, intermediate risk, high-risk, very high risk and for those with lymph node involvement, stage IV disease.[337] Patients with very low risk (PSA ≤ 10 μg/L, Gleason score ≤ 6 on biopsy, clinical stage T1c, fewer than three positive biopsies and ≤ 50% PCa in any core) and low risk PCa, (PSA ≤ 10 μg/L, Gleason score ≤ 6 on biopsy and clinical stage T1 – T2a) may opt for "active surveillance," radiation therapy or radical prostatectomy.[337]

Active surveillance involves postponing treatment with annual or biannual PSA and DRE monitoring. In active surveillance, treatment with curative intent is done on sign of progression and this is meant to decrease treatment related morbidities in low risk men who might never develop lethal PCa.[338] In contrast, monitoring of disease symptoms termed, "watchful waiting" is recommended for patients who do not have very long to live (per the American Urological Association (AUA), this would be less than or equal to five years), those with significant co-morbidities or those who do not wish to have curative treatments.[339] For men with local or regional disease at diagnosis, 81% and 12% respectively, single modality with radical prostatectomy (RP) or radiation therapy (RT; external beam or brachytherapy) are effective treatments with a five-year survival rate of 100%.[340]

However, treatment related morbidity including urinary incontinence, erectile dysfunction or declining bowel function result in a poorer quality of life.[341,342] Some of the side effects have

been ameliorated by use of nerve-sparing radical prostatectomy.[343] Other approaches for localized PCa include focal ablation therapy where cryotherapy, high-intensity focused ultrasound (HIFU), photodynamic therapy, or laser ablation are used to destroy a targeted region harboring PCa.[344] While focal ablation therapy is associated with diminished treatment related morbidity, its utility for long-term treatment remains to be seen.[344] Early PCa progression is driven by androgens making androgen ablation therapy (ADT) a mainstay treatment.[345,346]

ADT can be achieved by the surgical removal of both testicles (bilateral orchiectomy) which make testosterone or pharmacologically.[136] Testicular testosterone production is under the regulation of the hypothalamic–pituitary–gonadal (HPG) axis.[347] Hypothalamic Gonadotrophin-releasing hormone (GnRH) stimulates the pituitary gland to secrete Luteinizing hormone (LH) which triggers testicular testosterone production and in a negative feedback loop, testosterone dose dependently suppresses LH secretion.[347] Pharmacologically therefore, ADT can be achieved using luteinizing hormone-releasing hormone (LHRH) agonists and antagonists which block LH secretion in the pituitary via negative feedback or competitive inhibition and subsequently shut down testosterone production.[136]

Together with anti-androgens (*e.g.* bicalutamide and ARN-509) that competitively inhibit androgens from binding to AR and androgen synthesis inhibitors, (*e.g.* Abiraterone acetate and ketoconazole) that inhibit androgen production in the adrenal and prostate glands, these agents can completely suppress androgen signaling.[136,348] Treatments recommended for men with intermediate risk disease, (PSA of ≥ 10 – ≤ 20 µg/L, Gleason score = 7 and clinical stage T2b – T2c), include RT with either external beam radiation therapy (EBRT) or brachytherapy alone or combined with androgen deprivation therapy.[337] Alternatively, men with intermediate risk can

have an RP and a Pelvic Lymph Node Dissection (PLND) for initial therapy since metastases often involve lymph nodes and then adjuvant therapy with EBRT (plus or minus ADT).[337]

Men with high risk (PSA of ≥ 20 µg/L Gleason score = 8 – 10 and clinical stage T3a) and very high risk disease (PSA of ≥ 20 µg/L Gleason score = 8 – 10 and clinical stage T3b – T4) receive EBRT (alone or combined with a brachytherapy boost) and ADT or docetaxal chemotherapy.[337] For men with locally advanced disease without fixation to local tissues can receive radical prostatectomy with PLND.[337] Regional disease is treated with EBRT and ADT while metastatic disease is treated with ADT.[337]

PSA testing is used to monitor for recurrence after local therapy with biochemical recurrence characterized as a rise of 0.2ng/mL after RP or 2ng/mL after RT.[349] However, 20 – 40% RP and 30 – 50% RT patients experience biochemical recurrence within 10 years.[350-352] Depending on the initial local therapy administered and the whether the recurrence is local or metastatic, recurrent PCa is treated with salvage radiation therapy, salvage prostatectomy or ADT.[353] Eventually, however, most tumors become castration resistant (CRPC). Non-metastatic CRPC, is however still treated with ADT therapy because the AR pathway is found to be still active in these patients.[354] First-generation therapies include anti-androgens; flutamide, nilutamide and bicalutamide or inhibitors of androgen synthesis like ketoconazole with steroid.[354]

Enzalutamide, a second-generation androgen receptor antagonist approved for metastatic CRPC (mCRPC) is also used to treat non-metastatic CRPC.[354] Enzalutamide inhibits androgenic signaling by inhibiting AR nuclear translocation, chromatin binding, and coregulator binding.[355] Treatments for mCRPC treatment include androgen synthesis inhibitors, (Abiraterone acetate

plus prednisone or ketoconazole), first (bicalutamide, nilutamide, flutamide) and second generation AR blockers (enzalutamide), and chemotherapy (Docetaxel or Cabazitaxel).[354] Additionally, in 2010, Food and Drug Administration (FDA) approved an immunotherapy, Sipuleucel-T, for the treatment of asymptomatic or minimally symptomatic mCRPC.[356]

Sipuleucel-T uses a patient's autologous antigen presenting cells activated against a common PCa antigen (prostatic acid phosphatase) and linked to granulocyte-macrophage colony-stimulating factor (an adjuvant) thereby activating the host's PCa specific T cell response.[356] Unfortunately, CRPC treatments confer a median overall survival benefit of less than five months.[357-363]

Prostate cancer prevention

Given the drawbacks of screening, treatment associated morbidity, lack of effective treatment for advanced disease and several non-modifiable risk factors like age, ethnicity and genetics, effective PCa prevention strategies are imperative. Chemoprevention describes the use of natural, synthetic or biological substances to reverse, retard or inhibit the initiation of carcinogenesis or the progression of neoplasia to malignancy.[364] PCa has a slow progression; although early pathological changes like PIN are evident in men younger than 30 at autopsy, the median age of diagnosis with clinical disease is 66.[340,365] Similarly, PCa has a long latency within the gland before progressing through several stages of locally invasive, metastatic, and castration resistant disease.

The long natural-history of PCa offers a long window for preventive interventions. Like other carcinomas, PCa proceeds through the sequential accumulation of genetic and epigenetic

changes to the epithelium and the surrounding microenvironment transforming normal cells to malignancy and eventually metastasis.[366] As the disease progresses therefore, therapies would need to reverse many more molecular aberrations compared to early-stage disease, supporting the rationale for early prevention.[367] Additionally, late-stage interventions are bound to be compounded by co-morbidities which are more common in old age.[368] PCa chemoprevention is also an attractive public health strategy since reduction in disease incidence averts treatment associated morbidities along with the linked costs.[369]

Shortfalls of Pre-clinical Models of Prostate Cancer Chemoprevention

Two-dimensional tissue culture and organ-specific animal models are typically used to identify and test the efficacy, mechanism of action, dosing and safety of agents for the chemoprevention of specific forms of cancers.[370] Quantitative data gleaned from short-term mechanistic and biochemical *in vitro* assays serve as early indicators for chemoprevention efficacy for further evaluation in longer term assays and in whole animal models.[370] The efficacy, dose response, toxicity and pharmacokinetic data of promising agents are then tested in well established chemically induced, spontaneous or genetically engineered PCa models with the best candidates proceeding to clinical chemoprevention trials.[370]

However, although conventional tissue culture has advanced the conceptual understanding of PCa biology, it fails to recapitulate *in vivo* three-dimensional cell-cell and cell-matrix interactions, whose disruption plays a key role in cancer initiation and progression.[371] Unlike in 2D cultures, *in vivo*, cells in the tumor microenvironment (immune cells, fibroblasts, myofibroblasts, and endothelial cells), nutrient and oxygen gradients all regulate tumor

growth.[371] Furthermore, compared to other common cancers, there is a shortage of PCa cell lines as they are difficult to establish.[371] Moreover, majority of the *in vitro* studies prescreening primary chemopreventive agents utilized immortalized human PCa cell lines, like DU-145, PC-3 and LNCaP, which model advanced disease.

Preclinical models should however recapitulate the phase of disease progression under study and these studies should have used models of disease initiation or benign prostate epithelial cells instead. Ideal animal models for PCa chemoprevention also ought to reflect human PCa tumor biology as much as possible in terms of precursor lesions, course of progression, histology and molecular aberrations.[370] Additionally, the carcinogen or mutations used to generate the cancer should be relevant to the human disease and should generate a consistent tumor burden in a reasonable time frame.[370] In addition, the predictive accuracy value of the animal model for the clinical trial outcome should be ≥ 80%.[370] So ideally, murine PCa (mPCa) would originate from epithelial cells progressing to invasive adenocarcinoma through PIN.[372]

mPCa should also be androgen responsive like most human PCas and regress with ADT with recurrent tumors progressing to CRPC.[372] Metastases in mPCa tumors should also show the same tropism as human PCa which mostly metastasize to the bone.[372] However, no single model encompasses all these characteristics, and most murine models just recapitulate one or more stages of PCa progression.[372] Besides, considerable differences exist between human and murine prostates, which are some of the most popular PCa animal models. Whilst PCa is a disease of old men with slow progression, these features cannot be modeled in to mice without compromising the experimental design.[372] Furthermore, mice rarely spontaneously develop PCa unlike human

males who have a one in nine (1/9) lifetime risk of developing PCa pointing to differences in human and mouse prostate biology and tumorigenesis.[372]

Additionally, unlike single-organ human prostates, mice prostates have four distinct lobes calling into question which lobe is most representative of the human prostate.[372] Moreover, differences also exist between the mouse and human stroma.[372] The most commonly used animal models for PCa chemoprevention studies include; the transgenic adenocarcinoma of the mouse prostate (TRAMP), the LADY and PTEN-deficient mouse models and the Dunning rat model. In the TRAMP model, prostatic adenocarcinoma is driven by expression of the SV40 small and large T antigens under the androgen regulated rat probasin (PB) promoter, which inactivates the p53 and Rb tumor suppressors.[373] This model rapidly progresses to prostatic neoplasia at 28 weeks with 100% and 67% of the animals harboring lymph node and pulmonary metastases respectively.[374]

In the TRAMP model, 80% of the mice were castration resistant a phenotype mediated by differentiation to neuroendocrine tumors.[375,376] In the LADY, the large PB promoter drives the expression of the SV40 large T antigen with a truncated small T antigen distinguishing it from TRAMP.[372] Prostatic changes in LADY mice progress somewhat slower than in the TRAMP with mice PIN (mPIN) with limited adenocarcinoma developing by 15 – 22 weeks without metastasis to other organs.[377] Mouse models bearing similar genetic lesions as human PCa have also been used in chemoprevention studies. Loss of the PTEN tumor suppressor is a common event in PCa leading to preclinical chemoprevention studies using mice with Pten heterozygous deletion often in combination with other genetic common PCa lesions like Nkx3.1 haploinsufficiency.[378,379] However the Nkx3.1; Pten double heterozygous mice mimic key features of advanced PCa.[380]

Criticisms against the use of these mice models for primary chemoprevention include: The quick progression of mPCa in the TRAMP mice compared to slow progressing human PCa.[372] In the TRAMP and LADY models, mPCa is driven by viral oncogenes irrelevant to human PCa.[372] TRAMP mice mPCas also develop a neuroendocrine phenotype typically observed in a small subset of advanced PCas making it a poor model for PCa initiation that is also irrelevant for the majority of patients.[372,381] Additionally, the genetic changes driving prostate tumor formation in all three models, loss of p53, Rb and PTEN typically occur in advanced human PCas.[382] Primary chemoprevention models should ideally only contain genetic lesions that occur early in PCa development and have slow progression like that seen in human PCa.

Ironically, while several early-stage tumorigenesis models have been developed, they have not been popular in chemoprevention studies.[383] Other chemoprevention models include the Dunning rat model whose spontaneous and slow-growing prostate tumors have been used to generate cell lines for use in orthotopic xenograft models to test inhibition of disease progression.[384] Chemoprevention studies have also utilized rat models whose prostate tumors were induced using hormones, like the NBL (or Noble) steroid hormone rat model, or carcinogens, like the N-Nitroso-N-methylurea (NMU) rat model.[385,386] As dogs develop spontaneous prostate tumors with aged dogs exhibiting HGPIN lesions and adenocarcinoma, canine models could be relevant for PCa chemoprevention studies.[383] However, the development of canine chemoprevention models has been hampered by the high cost and genetic heterogeneity.[383]

Chemoprevention strategies

<u>Targeting the AR axis</u>

Since PCa is linked to androgen signaling, two chemoprevention trials tested 5α-reductase inhibitors (5ARIs) finasteride and dutasteride, which decrease 5α-dihydrotestosterone (DHT), the most active form of androgen. Finasteride and dutasteride reduced the risk of low grade PCa by 25% and 23% in the Prostate Cancer Prevention Trial (PCPT) and the Dutasteride of Prostate Cancer Events (REDUCE) trials respectively.[387,388] These drugs were not approved by the FDA for PCa prevention however due to a slight increase in the risk of high-grade PCa.[389]

Targeting oxidative stress

Oxidative stress has been linked to prostate carcinogenesis, propelling the use of dietary antioxidant supplements including vitamin E and selenium in PCa chemoprevention studies.

Basis for Selenium and vitamin E supplementation

Vitamin E preclinical findings:

Vitamin E refers collectively to four different isomers of tocopherols and tocotrienols, α, β, γ and δ that differ in the degree of methyl substitution on their chroman rings and often with differing biological activity.[390,391] Naturally occurring α-tocopherol has an RRR-configuration at the 2', 4', and 8' chiral carbons (RRR-α-tocopherol).[390] Synthetic α-tocopherol esters (acetate or succinate) contain an equimolar mix of all the eight different stereoisomers arising at the three chiral centers (all-racemic, or all-rac-α-tocopherol) and are used fortify food and in supplements.[390] RRR-α-tocopherol is said to be more biopotent than all-rac-α-tocopherol probably because some of the isomers in the latter are inactive.[392]

Interestingly, even different esters are shown to have different bioactivities with α-tocopherol-succinate having a distinct anti-prostaglandin effect relative to the acetate ester or (RRR-α-tocopherol) in human lung epithelial cells.[393] Both α and γ tocopherol exhibit anti-tumorigenic effects but due to its ability to scavenge both ROS and reactive nitrogen species (RNS), γ-tocopherol is said to be superior to α-tocopherol.[394-396] *In vitro*, α-tocopherol is anti-proliferative and apoptotic whereas α-tocopherol-succinate (Vitamin E succinate- VES) induces G1 cell-cycle arrest and decreases expression of anti-apoptic proteins XIAP, Bcl-XL and Bcl2 in PCa cells.[397-400]

Besides, overexpression of the alpha tocopherol transfer protein (α-TTP), which specifically facilitates α-tocopherol transportation in to plasma membranes, sensitizes PCa cells to the anti-proliferative effects of α-tocopherol by suppressing intracellular ROS.[401,402] The analog RRR-alpha-tocopheryloxybutyric acid (TOB) and VES reduce cell viability, induce apoptosis and disrupt AR signaling, which is indicated by the decreased expression of the AR target, PSA in PCa cell lines.[403,404] VES and α-tocopherol acetate trigger JNK signaling and upregulate the expression of Fas and FasL, which activates extrinsic and JNK cell death pathways in PCa cell lines but not in normal human prostate epithelial cells (PrEC).[405,406]

VES also induces the antiproliferative and proapoptotic insulin-like growth factor binding protein-3 (IGFBP-3), reducing growth of PCa xenografts *in vivo* and slowing tumor progression in TRAMP mice.[407] VES inhibits NFκB and cell adhesion molecules suppressing a metastatic phenotype in PCa cell lines.[398] However, a study in chicken broilers showed that α-tocopherol acetate is better for use *in vivo* as hydrolysis and poor absorption led to lower tissue concentrations of VES in tissues.[408] Additionally, whereas a different vitamin E analogue, RRR-

alpha-tocopheryloxybutyl sulfonic acid (VEBSA), had similar antiproliferative and apoptotic activities on PCa cells *in vitro*, it had a much higher activity than VES *in vivo* against tumor xenografts and disease progression in TRAMP mice.[409]

Oral administration of all-rac-α-tocopherol also inhibited growth of prostate tumor xenografts in nude mice on a high-fat but not low-fat diet implicating dietary fats and oxidative stress on disease progression.[410] However, intraperitoneal injections of VES significantly reduced the growth of prostate tumor xenografts in nude mice on both low and high-fat diets suggesting that the route of administrating affects vitamin E activity.[411] Oral administration of γ-tocopherol induced apoptosis and reduced PCa progression in a dose-dependent manner in the Transgenic Rat for Adenocarcinoma of Prostate (TRAP) model.[412]

In TRAMP mice, a tocopherol mixture enriched in γ-tocopherol significantly reduced tumor growth and reduced the incidence of HGPIN.[413] Supplementation with a γ-T-rich mixture of tocopherols reduced the development of mPIN lesions and DNA damage in CYP1A-humanized (hCYP1A) mice with chemically induced PCa using PhIP, a dietary carcinogen.[414] α and γ-tocopherol specifically dephosphorylate Akt at Ser473 by co-recruiting Akt and the phosphatase PHLPP1 to the plasma membrane through PH domain recognition.[415] α and γ-tocopherols that were structurally optimized for Akt and PHLPP1 recruitment, α-VE5 and γ-VE5 respectively, had increased Akt Ser473 dephosphorylation activity causing more apoptosis to PCa cells *in vitro* and anti-tumorigenic effects to PCa xenografts.[415]

γ-tocopherol but not α-tocopherol synergized with δ-tocopherol to inhibit PCa but not normal cell growth *in vitro* and induced apoptosis in androgen sensitive PCa cells by interrupting *de*

novo sphingolipid synthesis.[416] δ-tocopherol induced apoptosis more efficiently in human PCa cells *in vitro* than α-tocopherol and was antitumorigenic against tumor xenografts *in vivo*.[417] In prostate specific Pten$^{-/-}$ mice, δ-tocopherol but not α-tocopherol inhibited pAKT(T308) which slowed PCa progression by reducing proliferation and inducing apoptosis.[418] Interestingly, the Pten$^{-/-}$ mice did not have oxidative stress in the course of PCa progression.[418]

Reduction in the incidence of epithelial dysplasia in rat ventral prostates of N-methyl-N-nitrosourea (MNU) induced PCa by γ-tocopherol was attributed to suppression of cell proliferation, MMP-9 activation as well as (GST-pi) and Cox-2 immunoexpression.[419]

Selenium preclinical findings

Selenium (Se), an essential trace element, is incorporated in to more than 25 selenoproteins a special tRNA.[420] Some selenoproteins like glutathione peroxidases (GPx) and Thioredoxin reductases (TRs) have antioxidant activity.[421] Therefore, inorganic (selenite and selenite) and organic forms (Methylseleninic acid (MSA) and selenomethionine (SeMet)) of selenium have been tested for anti-cancer effects. High doses of sodium selenite paradoxically induced oxidative stress in LNCaP cells with short-term and long-term treatments causing apoptosis and cell cycle arrest respectively.[422] In DU145 PCa cells, selenium slowed cell growth and induced extrinsic and intrinsic apoptotic pathways by increasing; death receptor 5 (DR5) expression, caspase 8 activation and Bid cleavage.[423,424]

In several cancer cell lines, including DU-145, SeMet caused dose-dependent growth inhibition, aberrant mitosis and apoptosis.[425] High local concentrations of MSA inactivated mitogenic protein kinase C isoenzymes (PKC) whose cysteine sulfhydryls in the catalytic domain

underwent catalytic oxidation inhibiting growth and inducing apoptosis in DU145 cells an effect that is reversed by the selenoprotein Thioredoxin reductase (TR).[426] High TR expression in PCa cells correlated with MSA resistance and auranofin, a TR specific inhibitor sensitized PCa cells to MSA indicating that levels of PKCε and TR might modulate the effects of selenium.[426,427]

Sodium selenite and MSA prevent NFκB activation induced by inflammatory stimuli by inhibiting IκB-α degradation reducing cell growth and inducing apoptosis in DU145 and JCA1 PCa cells.[428] In LNCaP cells, high concentrations of MSA decreased the expression of NFκB and disrupted its DNA binding, which decreased the expression of antiapoptotic and pro-inflammatory genes NFκB target genes.[429] MSC (Methyl selenocysteine) inhibited proliferation, colony formation and induced apoptosis in DU145 cells by upregulating connexin 43 (Cx43), downregulating Bcl-2 and up-regulating Bad expression.[430] MSA led to detachment of DU145 cells and loss of attachment (LOA) cell death (anoikis) partly through Caspace 8 activation whereas sodium selenite led to apoptosis through JNK and p38.[431]

Sodium selenite and SeMet caused dose-dependent inhibition of proliferation and anchorage independent growth and induced apoptosis in PCa cells but not normal primary prostate cells with sodium selenite being more potent.[432] SeMet increased the phosphorylation of Tyr15 in the p34cdc2 (cdk1) kinase, causing G2-M cell cycle arrest.[432] Low concentrations of MSA increased the expression of p27kip1 and p21cip1 by upregulating the zinc-finger transcription factor Krüppel-like factor 4 (KLF4) causing G1 cell cycle arrest in PCa cells; higher MSA concentrations also induced apoptosis attributed to reduction in pAKT and pERK1/2.[433-435] In contrast, sodium selenite decreased p27kip1 and p21cip1 expression and induced caspase-independent cell death by increasing JNK1/2, and p38MAPK phosphorylation.[433]

SeMet increased the expression of Cip1/p21 and Kip1/p27 leading to a G1 cell cycle arrest in androgen sensitive cells but not in the AR-null cell line, PC3.[436] AR expression in PC3 cells led to a G2/M cell cycle arrest indicating that the effects of SeMet were dependent on a functional AR.[436] In LNCaP cells, MSA and methylselenol enhanced the recruitment of AR corepressors reducing the expression of AR target genes, decreased AR mRNA stability and nuclear localization.[437-440] In contrast, sodium selenite inhibits AR expression through a superoxide mediated redox mechanism, and by inhibiting the DNA binding of the AR transcription factor SP1, it also inhibits IL6 mediated AR activation by upregulating c-Jun.[441,442]

SeMet decreases proliferation of tumor adjacent 'normal' primary prostate cells with a concurrent decrease in AR signaling.[443] Sodium selenite selectively inhibits proliferation and induces p53 dependent and independent apoptosis of human PCa but not normal cells.[444,445] Metabolites of the polyunsaturated fatty acids (PUFAs) metabolic enzyme, arachidonate 5-lipoxygenase (ALOX5), or their precursor arachidonic acid could rescue sodium selenite's apoptotic phenotype.[444] Sodium selenite therefore induces apoptosis partly by disrupting the metabolism of arachidonic acid a common PUFA in high fat Western-style diets that is associated with PCa cell proliferation.[444,446-448]

PCa cells differed in their sensitivity to the effects of different forms of selenium depending on their PTEN status due to differential effects on pAKT activity and distinct effects on pERK1/2 activity.[449] MSA induced ER stress and an unfolded protein response (UPR) in p53-null PC-3 cells by disrupting oxidative protein folding culminating in p21(WAF) dependent growth arrest and apoptosis.[450,451] Sodium selenite increased p53 activity and p53 dependent superoxide generation from sodium selenite metabolism, which coupled with decreased expression of

dismutase enzymes MnSOD and SOD selectively induced apoptosis in human prostate tumors but not the matching normal tissue and sensitized p53 wild type PCa cells to apoptosis.[452-455]

In PTEN deficient PC3 cells, selenium decreased PI3K activity attenuating PIP3 levels and membrane recruitment of AKT and its activating kinase PDK1 decreasing pAKT (Thr308).[456] Selenium also decreased pAKT (Ser473) in a calcineurin (a calcium-dependent phosphatase) dependent manner.[456] SeMet and MSA activated overlapping and distinct gene transcription programs involved in cell cycle regulation, apoptosis and androgen signaling in LNCaP cells suggesting different forms of selenium have unique cellular effects.[457] Selenite decreased levels of the DNA methyltransferase 1 (DNMT1) causing partial promoter demethylation leading to a dose- and time-dependent re-expression of the detoxifying enzyme, π-class glutathione-S-transferase (GSTP1) in LNCaP cells.[458]

In DU145 cells, sodium selenite increased PTEN phosphorylation at Ser370 in a dose dependent manner probably by increasing the activity of casein kinase-2 (CK2) which decreased p-AKT(Ser473).[459] In PCa cells, MSC but not selenite decreased levels of several types of collagen including collagen type I alpha 1 (COL1A1), COL1A2 , COL7A1, COL6A1 and COL4A5 indicating that anticancer cancer effects of MSC might result from extracellular matrix modulation.[460] MSA inhibited the expression of the antiapoptotic protein survivin in LNCaP cells by preventing SP1 promoter binding, while MSA did not alter survivin levels in castration resistant PCa cells, its knockdown and MSA treatment had a synergistic antiproliferative and apoptotic effect.[461,462]

In LPS-stimulated PC3 cells, sodium selenite inhibited NFκB nuclear translocation, reducing the expression of TGFβ1 an immunosuppressive cytokine, VEGF an angiogenic factor and the pro-inflammatory factor IL-6.[463] In hypoxic conditions, MSA induced apoptosis and inhibited growth in castration resistant PCa cells by decreasing the expression of HIF-1α and increasing the expression of REDD1 (regulated in development and DNA damage response-1), which is a negative regulator of mTOR.[464,465] In nude mice, sodium selenite retarded the growth and progression of castration resistant PC3 orthotopic tumors and lymph node metastases associated with anti-angiogenic activity.[466] *In vivo*, MSA and MSC had a superior dose-dependent growth inhibition of DU145 and PC-3 human PCa xenografts compared to SeMet and selenite in spite of lower tumor retention.[467]

MSA and MSC induced apoptosis, inhibited proliferation, decreased circulatory levels of insulin-like growth factor 1 (IGF1), delayed PCa progression and increased survival in the TRAMP mouse model.[468] MSC reduced tumor growth in the Dunning model of rat prostate cancer through unknown mechanism(s) while Vitamin E did not.[469] MSA reduced the incidence of HGPIN and PCa progression in a Pten prostate-specific knockout (KO) mice by inducing senescence through P53 activation and attenuation of pAkt and AR signaling.[470] In LADY transgenic mice, a mix of vitamin E, selenium, and lycopene decreased proliferation and inhibited development of PCa with increased survival.[471] Sodium selenite and MSA decreased the growth of androgen-dependent LAPC-4 and LNCaP tumor xenografts respectively with a concomitant decrease in AR and PSA expression.[472,473]

However, some studies have found null activity for selenium and vitamin E *in vivo* against PCa. Neither SeMet nor alpha-tocopherol inhibited PCa development in the testosterone plus

estradiol-treated NBL rat model where prostate tumorigenesis is driven by sex hormone-induced oxidative stress and inflammation.[474,475] Supplementation with SeMet and/or α-tocopherol did not decrease PCa incidence in Wistar-Unilever rats with N-methyl-N-nitrosourea (MNU) induced PCa followed by chronic stimulation with androgen.[476] However, some studies have indicated that there is a synergistic effect for selenium and vitamin E. A combination of MSA and γ-Tocopherol decreased growth of tumor xenografts by upregulating the expression of proapoptotic proteins Bax and Bad and downregulating pro-survival Bcl2.[477]

MSA and VES (but not α-tocopherol acetate) activated separate cell death pathways in PCa cells suggesting synergistic chemoprevention.[478] Flow cytometry shows that vitamin E and selenium reduce the number of cycling LNCaP cells presumably by leveraging distinct mechanistic pathways.[479]

Vitamin E and Selenium clinical trial findings:

Support for use of vitamin E in a large PCa chemoprevention trial came from secondary analysis of the Alpha-Tocopherol, Beta-Carotene Cancer Prevention Trial (ATBC).[480] ATBC tested the efficacy of 50 mg daily of α-tocopheryl acetate and/or 20 mg daily of β-carotene supplementation for a median of 6.1 years on prevention of lung and other cancers in 29,133 male smokers aged 50–69 years in Finland.[481] Unexpectedly, β-carotene increased risk of lung cancer and total mortality whereas α-tocopherol had a null effect.[481] However, there was a 32% and 41% reduction in PCa incidence and mortality respectively among men on α-tocopherol which was inversely correlated with serum α-tocopherol levels especially in advanced disease.[482,483]

Nevertheless, this beneficial α-tocopherol effect disappeared post-intervention.[484] In the VITamins And Lifestyle (VITAL) cohort study, long term vitamin E or selenium supplementation had a null effect on overall PCa risk though there was a 57% reduction in risk of advanced PCa among men on vitamin E.[485] An earlier study on 47,780 healthy men in the U.S found that at least 100 IU supplemental vitamin E daily lowered risk of metastatic and fatal PCa but only in smokers or recent quitters.[486] Analysis of 72,704 men in the Cancer Prevention Study II Nutrition Cohort found no association between regular vitamin E supplementation and overall risk of PCa or advanced PCa, although there was a slight risk reduction in smokers.[487]

Selenium was first demonstrated to be protective against liver, stomach and esophageal cancers in large randomized trials in Qidong and Linxian, China.[488] A general population interventional trial with selenized table salt for eight years in Qidong County (n= 130,471) where hepatitis B virus (HBV) infection and liver cancer are prevalent reduced the incidence of liver cancer by 35% an effect that reversed by ceasing supplementation.[489] A smaller interventional trial (n= 226) in the same region found that supplementation with selenized yeast for four years reduced liver cancer cases among people positive for Hepatitis B Surface Antigen compared to the placebo an effect that reversed upon cessation of supplementation.[489]

The Linxian Nutritional Intervention Trials tested whether supplementation with multiple vitamins and minerals would decrease the risk of cancer and cancer mortality.[490,491] The first trial found significantly reduced overall mortality among individuals on carotene, inorganic selenium and vitamin E attributed to a decline in cancer incidence in the general population.[490] However, a second trial found that β-carotene, inorganic selenium and vitamin E had a null effect on esophageal cancer incidence and mortality in people with esophageal dysplasia.[491] Enthusiasm

for use of selenium in a PCa chemoprevention trial came from findings of the Nutritional Prevention of Cancer (NPC) study.[480]

In this trial, 1,312 participants (74%) male with a prior history of non-melanoma skin cancer were randomized to receive 200 μg daily dose of selenium from selenized yeast or placebo for 4.5 ± 2.8 years and followed for 6.4 ± 2.0 years for the development of non-melanoma skin cancer and other cancers.[492] Although the brewer's yeast had no effect on the primary endpoint, there was a 63% reduction in risk of PCa as a secondary end point.[492] Additional stratified analyses found the reduction in PCa risk to be greater in men with low baseline selenium levels.[493,494]

The Selenium and Vitamin E Cancer Prevention Trial (SELECT)

In 2001, the NCI initiated the SELECT trial, which enrolled 35,533 men over the age of 50 years, to test the chemopreventive benefit of selenium and vitamin E on prostate cancer (PCa) with an intended follow up of 12 years.[480,495] As part of the inclusion criteria, the men had to be free of a prior PCa diagnosis, have a non-suspicious digital rectal examination (DRE) and a PSA ≤ 4ng/mL.[480] SELECT, a phase III, double-blinded, prospective, 2x2 factorial clinical trial randomized participants to a daily dose of 200 μg L-selenomethionine, 400 IU α-tocopheryl acetate, a combination of both agents at the same dosage or placebo.[480,495]

The intended 12-year study was powered to detect a 25% decline in PCa incidence on single agent therapy and an additional 25% reduction with the combined therapy compared to the placebo.[480] However, an interim analysis after 5.5 years median follow up found no benefit for selenium or the combined therapies and a marginal increased risk of PCa in the vitamin E group

leading to early termination of the trial.[495] An additional 54,464 person-years of follow-up showed a 17% increased risk of PCa with vitamin E (P = 0.008).[496] A recent case-cohort study on SELECT participants tested whether the outcome of selenium and vitamin E supplementation on PCa risk depended on baseline selenium status.[497]

In the placebo group, toenail selenium, a reflection of long-term selenium exposure, was not associated with PCa risk.[497] Additionally, selenium supplementation, alone or in combination with vitamin E did not affect the PCa risk in men with low baseline selenium status.[497] However, in men with higher baseline selenium status, Selenium increased the risk of high-grade PCa by 91% (P = 0.007).[497] On the other hand, vitamin E supplementation (alone) had no effect on PCa risk in men with high selenium status but it increased the risk of total (63%, P = 0.02), low-grade (46%, P = .09), and high-grade PCa (111%, P = .008) in men with lower selenium status.[497]

Thus, whereas there was no benefit of selenium supplementation in men with low baseline selenium status, those with high baseline status had increased risk of high-grade disease.[498] Besides, vitamin E increased PCa risk in men with low baseline selenium status.[498] A separate phase III clinical trial in 699 men found selenized yeast supplementation to have no effect on the incidence of PCa in men at high risk for PCa (PSA > 4 ng/ml and/or suspicious DRE and/or PSA velocity > 0.75 ng/ml/year but negative PCa biopsy).[499]

Summary

The outcome of the SELECT trial remains a puzzle. As detailed above, numerous *in vitro* studies suggest that vitamin E and selenium have anticancer effects by inhibiting proliferation, altering redox homeostasis, inducing apoptosis and blocking inflammatory signaling. The lack of

efficacy and even harmful effects of vitamin E and selenium in large randomized clinical trials points to the low predictive value of PCa chemoprevention outcomes for *in vitro* and *in vivo* models referenced in the design of SELECT. Although primary prevention studies call for models of disease initiation, most of the preclinical studies described above used advanced PCa models.

Interestingly, several studies summarized above reported a null effect for vitamin E and selenium on normal prostate cells.[405,416,432,444,452] Furthermore, the VITAL study found that vitamin E and selenium supplementation had no effect on the risk of latent or early PCa but long-term vitamin E supplementation reduced the risk of advanced PCa.[485] Therefore, we hypothesized that prostate cells at different stages of cancer evolution process may respond differently to antioxidants. In addition, some have attributed the lack of efficacy in SELECT to the doses and formulations of vitamin E/selenium used.[500,501] SELECT tested a daily dose of vitamin E, (α-tocopheryl acetate; 400 mg), and/or selenium (L-selenomethionine; 200 μg).[480]

The ATBC trial had however demonstrated efficacy for a much lower dose of 50 mg α-tocopheryl acetate whilst the NPC trial had used 200 μg selenized yeast.[482,493] Furthermore, vitamin-E isomers have different bioactivities. Due to its ability to scavenge both ROS and reactive nitrogen species (RNS), γ-tocopherol is superior to α-tocopherol.[394] We tested the hypothesis that different vitamin E isomers and even an unrelated, water soluble antioxidant would have different effects *in vitro*. Moreover, the vitamin E and selenium *in vitro* studies prior to SELECT utilized conventional 2D culture.

Although 2D tissue culture has been useful in unravelling PCa biology, important limitations restrict its utility. Cells in 2D lack physiological cell and matrix interactions and attachment to artificial surfaces affects cell morphology and signaling.[502] Additionally, lack of oxygen and nutrient gradients in 2D cultures makes the environment non-physiologically uniform.[503] As much as the use of animal models overcomes some of these limitations, the systemic and physiologic differences between mouse and human prostates can affect phenotype.[371]

In addition, genetically engineered mouse (GEM) models are expensive and also take long to generate whereas xenograft models are limited by the small number of available PCa cell lines.[371] Moreover, the intractability of whole animal models makes them less ideal for investigating molecular mechanisms at the cellular level necessitating cell cultures. [504] As detailed previously, vitamin E and selenium supplementation studies in animal models produced mixed results with several studies showing no effect.

Given the shortcomings of 2D cell cultures and the difficulty of modeling the spectrum of human prostate tumorigenesis *in vivo*, we modeled different stages of PCa progression in 3D organoids and tested the effects of the SELECT supplements on PCa tumorigenesis. In three-dimensional (3D) cultures, cells form cell-cell and cell-matrix attachments mimicking an *in vivo* environment.[505] Additionally; growth factor, nutrients and oxygen gradients in 3D cultures yield heterogeneous cell populations like *in vivo.*[505]

CHAPTER 2: MATERIALS AND METHODS

Ethics statement

As previously published, all human subject research adhered to ethical standards, and all experiments on human samples were performed in accordance with stipulated guidelines and regulations.[506,507] Benign human primary prostate epithelial cells were acquired after approval from the University of Illinois at Chicago (UIC) Institutional Review Board (IRB) and informed consent obtained from all patients.[506,507]

Prostate tissue collection and cell preparation

Prostate tissue was obtained from radical prostatectomy PCa patients at the UIC Medical Center with informed consent and in accordance to IRB guidelines as previously described.[506,507] Benign tissue from the peripheral zone was resected, and a final H&E histological assessment done on a thin slice of tissue by a pathologist.[506,507] Only tissue confirmed to be 100% benign was utilized.[506,507] Prostate epithelial cells were isolated using a method developed by Donna Peehl.[508] In brief, tissue was digested with collagenase and primary epithelial cells (PrE) isolated by plating on collagen-coated dishes in serum-free prostate epithelial cell growth medium (PrEGM) media (Lonza no. CC-3166 & CC-4177).[506,507] qRT-PCR for expression of known basal epithelial cell markers (CK5+/p63+/AR-) confirmed the cell type.[506,507]

Cell culture

In preparation for organoid culture, 50,000 PrE cells from two patients were plated in 10 cm dishes in primocin (Invitrogen no. ant-pm-1) containing PrEGM media as described by Unno *et al*.[509] To expand them, the cells were passaged once at 50-70% confluence and used for organoid culture on the second passage at 80% confluence.[509] LNCaP cells were cultured in RPMI 1640 media (Gibco Life Technologies no. 11875-093) with 10% fetal bovine serum (FBS) - (Life Technologies no. 10437-028) and 1% Pen Strep antibiotic solution (Life Technologies no. 15140-122). RWPE-1 cells were cultured in keratinocyte serum-free media with 0.05 mg/ml bovine pituitary extract, 5 ng/ml epidermal growth factor (Thermo Fischer Scientific no. 17005042) and 1% Pen Strep antibiotic solution. Cells were tested and treated for mycoplasma and cell lines genetically authenticated by short-tandem repeat (STR) profiling.

Organoid culture and treatments

After expansion in their optimum media, the different cell types were trypsinized and transferred to organoid media as previously described.[509] Briefly; 5000 cells were resuspended in organoid media containing low percentage matrigel (5%) then plated in to 96-well ultralow attachment plates (Corning no. 3474). A hundred microliters of fresh media was added to the cultures every four days or every two days once the treatments commenced. Treatments were added to the following final concentrations; 40 μM DL-αTocopherol-Acetate (Sigma no. T3376), 40 μM RRR-γ-Tocopherol (Sigma no. T1782) and/or 1.3 μM Seleno-L-methionine (Sigma no. S3132) representing the mean concetrations attained in the blood plasma of the SELECT subjects.[495] N-acetyl cysteine (NAC; Sigma no. A9165) and Etomoxir sodium salt hydrate (Eto; Sigma no. E1905) were used at various concentrations as indicated in the figures. Organoid growth was

captured by brightfield microscopy using Zeiss Axioskop/Nuance microscope (Carl Zeiss Inc. Oberkochen, Germany).

2D Cell Proliferation Assay

To monitor the effect of the SELECT supplements on cell proliferation in 2D, replicates of pretreated RWPE-1 (n = 6 – 9) were plated in 96-well plates at densities of 2000 cells/well. The plates were scanned with the IncuCyte ZOOM™ live cell imaging system (Essen BioScience) with continued treatments. Images were captured every four hours for the durations indicated using the 10x objective. Cell confluence was calculated with the IncuCyte ZOOM™ software (version 2015A).

High Throughput Sequencing

A high throughput strategy targeting 222 cancer related genes was used to sequence DNA extracted from human prostate epithelial cells used for organoid culture to ascertain that they did not contain PCa relevant mutations. DNA Probes for capturing exon regions in these genes were manufactured by Roche NimbleGen. SeqCap EZ Library SR User's Guide (Roche, Pleasanton, CA) was followed for library preparation and capture of targeted sequences. Paired-end sequencing of 2 x 150 bp was performed on a MiSeq platform (Illumina, San Diego, USA). Twelve individual libraries were multiplexed for a MiSeq flow cell. The mean sequencing depth of coverage was 135x.

Bioinformatics analysis

Paired-end reads were aligned to the GRCh37 version of the human genome using Burrows-Wheeler Aligner v0.7 to generate BAM files.[510] The BAM files were sorted with samtools, PCR duplicates marked using Picard and realignment around putative gaps performed using the Genome Analysis Toolkit (GATK) v3.2-2. The GATK Haplotype caller was used for variant calling. ANNOVAR (http://annovar.openbioinformatics.org/en/latest) was used to annotate variants as well as retrieving variant information from population-based studies such as the 1000 Genomes Project (www.1000genomes.org), NHLBI-ESP 6500 exomes or ExAC (http://exac.broadinstitute.org/), and clinical databases like the Human Gene Mutation Database (HGMD) and ClinVar. [511,512]

Variant pathogenicity was defined based on the American College of Medical Genetics and Genomics (ACMG) criteria.[513] Specifically, pathogenic and likely pathogenic mutations are defined as 1) all protein-truncating mutations unless their allele frequency is 5% or higher in any racial group in population databases or is reported as benign or likely benign in the ClinVar, and 2) nonsynonymous changes if their allele frequency is less than 5% and reported as pathogenic and likely pathogenic mutations in the ClinVar. The high throughput sequencing and analysis were carried out by the Genomics Core Facility at the NorthShore University Health System (Chicago, IL).

Histology and immunostaining

Fixation, processing, H&E and immunofluorescence staining were done as previously described.[509] The following primary antibodies were used: Ki-67 (1:100; eBioscience no.14-

5698-80), BrDU (1:100; Abcam no. ab6326), CK8 (1:400; Covance no. MMS-162P) and CK14 (1:500; Covance no. PRB-155P). The secondary antibodies (Life Technologies) used at 1:400 each were; goat anti-rabbit Alexa Fluor 647 (no. A21244), goat anti-rat Alexa Fluor 488 (no. A11006) and goat anti-mouse Alexa Fluor 568 (no. A11004). Sections were counterstained with 0.5 mg/ml DAPI (Sigma no. D-9542) and mounted in ProLong Diamond Antifade reagent (Molecular Probes no. P36961). When appropriate, BrDU (Invitrogen no. 00–0103) was added into the organoid medium at 1:100 dilution (3 μg/mL) overnight. BrdU was detected using a rat anti-BrdU antibody (1:100; Abcam no. ab6326). Imaging was performed using a Nikon A1R (A) Spectral laser scanning confocal microscopy (Nikon Instruments Inc. Yokohama, Japan, Japan).

Detection of Reactive Oxygen Species (ROS)

Mitochodrial ROS were detected using CellROX Green (Thermo Scientific C10444) according to the manufacturer's instructions. In brief, on day 21 of organoid culture, the probe was added to a final probe concentration of 5 μM. Staining was done in the dark for 1 hour at 37ºC. Organoids were washed in PBS and placed in chamber slides for imaging using the Nikon A1R (A) Spectral laser confocal microscope. The mean fluorescence intensity per image was determined using the Fiji (ImageJ) software.

Microarray analysis of antioxidant treated organoids

RNA was extracted using Trizol (Life Technologies No. 15596-026) from triplicates of organoids pooled from eight 96-plate wells. The RNA was cleaned up using an RNeasy Mini Kit

(Qiagen no. 74104) with DNAse (Qiagen no. 79254) on column treatment. The RNA was hybridized to Affymetrix HTA 2.0 transcriptome arrays and analyzed with the Affymetrix AGCC suite at the University of Illinois at Chicago (UIC) Genomics core (Chicago, IL). The CEL files were imported in to R (windows version 3.1.1) using the Bioconductor (version 3.3) oligo package. Raw intensity scores for probes were normalized by quantiles and background corrected with RMA. Differentially expressed genes (DEGs) were identified by the Bioconductor limma package. For functional analysis, the C2 (curated) gene sets of MSigDB (version 5.1) were queried using the Gene Set Enrichment Analysis (GSEA) algorithm.[514] Results were visualized with the Enrichment Map plug-in [version 2.0.1] on Cytoscape [version 3.2.1] using a p-value cutoff of 0.005, an FDR cutoff of 0.25, and an overlap coefficient cutoff of 0.5.[515,516]

Poly-HEMA coating and suspension cell cultures

We made a 1.5% solution of the anti-adhesive polymer, poly 2-hydroxyethyl methacrylate (Poly-HEMA; Sigma no. P3932), in 95% ethanol which we dissolved by rotating for several hours at 65ºC. We used 4 ml, 700 μL /well and 60 μL/well of the Poly-HEMA solution to coat 10 cm, 6-well and 96 well plastic culture plates respectively. The plates were left open overnight to dry and sterilized by UV for 45 minutes before use. Poly-HEMA reduces the adhesiveness of plastic surfaces in a concentration dependent manner.[517] While adherent cells in 2D plastic tissue culture are flat and stretched, cells cannot attach on poly-HEMA coated plates, keeping them in a suspension of spherically shaped cells.[517]

Glucose absorption assay

We plated 11,000 cells per well in 96-well plates with or without a 1.5% poly-HEMA coating (Sigma no. P3932). Media was collected after 24 h and diluted 1:2000 in water. The amount of glucose was measured using the Amplex Red Glucose Assay Kit (Thermo Fisher Scientific no. A22189) per the manufacturer's instructions.

ATP assay

Cells were plated in poly-HEMA coated or uncoated 96-well plates at a density of 11,000 cells per well. After 24 h, ATP was measured using the ATPlite Luminescence kit (PerkinElmer no. 6016943) per the manufacturer's protocol.

Oxygen consumption (OCR) and extracellular acidification rate (ECAR) measurements.

To measure the effects of the SELECT supplements on the energy metabolism of RWPE-1 cells, treatments were done for 5 days and switched to non-adherent conditions for 24 hours. We plated replicates of 30,000 cells/well (n = 15) in Celltak (Corning no. 354240) coated XF96 well Seahorse cell culture plates (Agilent no. 101085-004). An XF96 extracellular flux analyzer (Seahorse Bioscience) was then used to measure oxygen consumption and extracellular acidification rates. Oligomycin, Carbonyl cyanide 3-chlorophenylhydrazone (CCCP), Antimycin, Rotenone and 2-Deoxy-D-glucose (2DG) (Sigma) were injected to final concentrations of 5 μM, 0.75 μM, 2 μM, 2 μM, and 40 mM respectively. Experiments were performed in the DMEM based, XF-Base medium (Agilent Technologies no. 103335-100) without phenol red, bicarbonate, glucose or glutamine.

The medium was supplemented with 10 mM glucose (Sigma), 2 mM glutamine (Sigma), 5 mM HEPES (Sigma no. H0887), 2.5 µg human recombinant Epidermal Growth Factor and 25 mg Bovine Pituitary Extract (BPE) (Thermo Fisher Scientific no. 17005042). Basal OCR is the OCR value before the injection of any drugs and after the subtraction of the OCR values after the injection of antimycin A and rotenone (A/R) to discount non-mitochondrial OCR. Maximal OCR is the OCR value after the induction of respiration with CCCP subtracting the non-mitochondrial OCR. Basal and maximal ECAR are the sensitivity of the extracellular acidification rate before the injection of any drugs and the injection of oligomycin respectively. The ECAR value after glycolysis inhibition with 2DG is subtracted from both the basal and maximal rates to discount non-glycolytic ECAR.

Statistical analysis

Statistical analyses were performed using a two tailed Student's t-test, one-way or two-way Analysis of Variance (ANOVA) with Tukey's correction for multiple comparisons. All results are presented as mean ± Standard Deviation or Standard Error. P values ≤ 0.05 were considered significant.

CHAPTER 3: RESULTS

The SELECT supplements decrease proliferation and induce cell death in LNCaP cancer organoids

Laboratory studies showing anti-tumorigenicity of vitamin E or selenium in the prostate mostly utilized LNCaP, PC-3, and DU145 tumor cells. However, the failure of SELECT led us to hypothesize that the response to antioxidants may depend on disease stage. To test this, we first evaluated the effects of the SELECT agents on LNCaP prostate cancer cell organoids (Fig. 4A). The human prostate epithelium contains basal, luminal and neuroendocrine cells distinguishable by the expression of specific markers.[73] From the CK8+ staining and absence of basal cell markers, LNCaP yielded purely luminal organoids (Fig. 4B).

In agreement with previous reports, the antioxidants strongly decreased BrDU incorporation relative to the vehicle control (Figs. 4B & C).[400] Furthermore, whereas the cycling cells in the vehicle were dispersed throughout the organoids, indicating anchorage independent growth, those in the antioxidant treated organoids especially vitamin E were restricted to the outermost layer proximate to the ECM (Fig. 4B). Additionally, the antioxidants induced cell death especially in the inner, extra-cellular matrix (ECM) deprived cells (Fig. 4B) consistent with previous reports.[399]

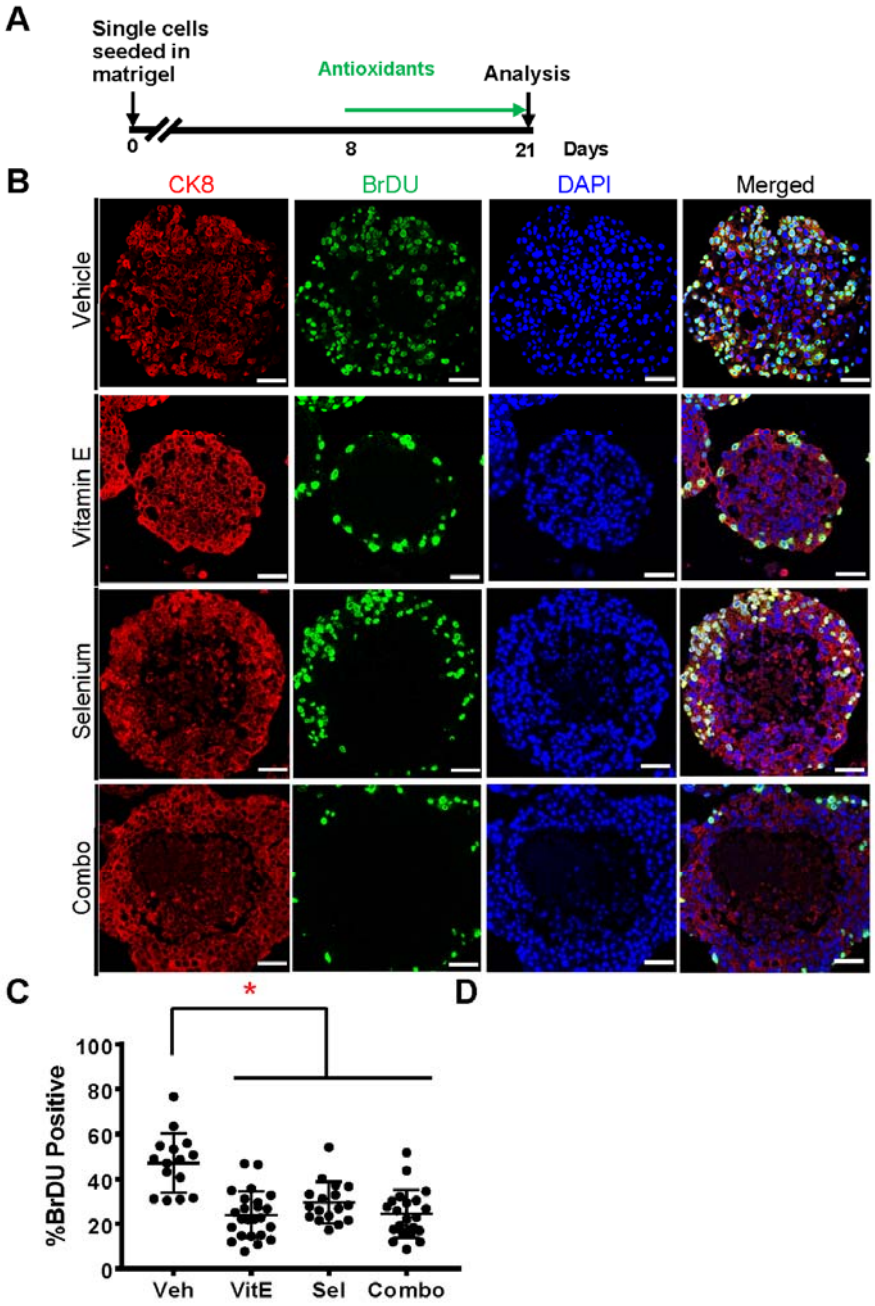

Figure 4. The SELECT antioxidants decrease proliferation and increase cell death in LNCaP organoids. LNCaP cells were grown in organoid media, treated with antioxidants then immunostained with anti-CK8, anti-BrdU antibodies and a DAPI counterstain (A) Time line of the culture and treatment of organoids (B) LNCaP cells gave rise to luminal organoids displaying a significant decrease in proliferation and increased cell death when treated with antioxidants. (C) Quantification of BrDU incorporating cells as a percentage of total cells shows that the antioxidants significantly reduced the number of actively dividing cells. Scale bars represent 50 μm. Asterisks represent statistical significance (One way ANOVA with Tukey's correction for multiple comparisons). *$p \leq 0.0001$; error bars represent SD.

The SELECT supplements do not increase cell proliferation in benign primary prostate organoids

Next, we tested the effects of the supplements on benign primary human prostate epithelial cell organoids. We generated organoids using benign primary prostate epithelial cells from two African American subjects as previously reported.[509] The absence of prostate cancer related alterations after the targeted sequencing of 222 cancer genes confirmed the cells to be benign (Table 1). The primary organoids were heterogeneous in size, morphology and expression of basal or luminal markers (Fig. 5A). Notably, antioxidant monotherapies did not affect the proliferation of these organoids whereas the combination treatment decreased Ki67 staining in the first subject (Figs. 5B & C).

Vitamin E significantly increased cell proliferation in premalignant RWPE-1 prostate organoids but not in 2D culture

The SELECT trial revealed a deleterious effect of vitamin E on a fraction of individuals without prior evidence of prostate cancer.[496] Therefore we reasoned that these individuals might have harbored "initiated" cells in a pre-malignant state that were pushed to malignancy by chronic antioxidant treatment. We tested this on organoids generated from the immortalized but non-tumorigenic RWPE-1 human prostate epithelial cell line. RWPE-1 cells are immortalized with the E7 oncoprotein from HPV18 which modulates the activity of the retinoblastoma (Rb) tumor suppressor.[518,519] Whereas most RWPE-1 organoids treated with the vehicle or selenium had hollow lumens, those treated with vitamin E or the combination displayed marked luminal filling (Fig. 6A).

Vitamin E induced a near two fold increase in proliferation with 35% Ki67 positivity compared to the vehicle at 19% (Fig. 6B). In contrast, selenium had no impact on proliferation whereas the combination treatment had an intermediate effect at 18% and 25% Ki67 positivity respectively (Fig. 6B). Confirming these results, vitamin E had the highest number of BrDU incorporating cells at 37%, however, Selenium (24%) and not the vehicle (27%) had the lowest (Fig. 6C). These *in vitro* RWPE-1 organoid findings where vitamin E enhances proliferation whereas selenium counteracts vitamin E, are highly reminiscent of the clinical trial data from SELECT. In contrast, vitamin E had no significant effect on the growth rate of RWPE-1 cells grown in 2D (Fig. 7). In this condition, the combination of vitamin E and selenium significantly increased cell growth (Fig. 7) indicating that 2D culture could not recapitulate the SELECT results.

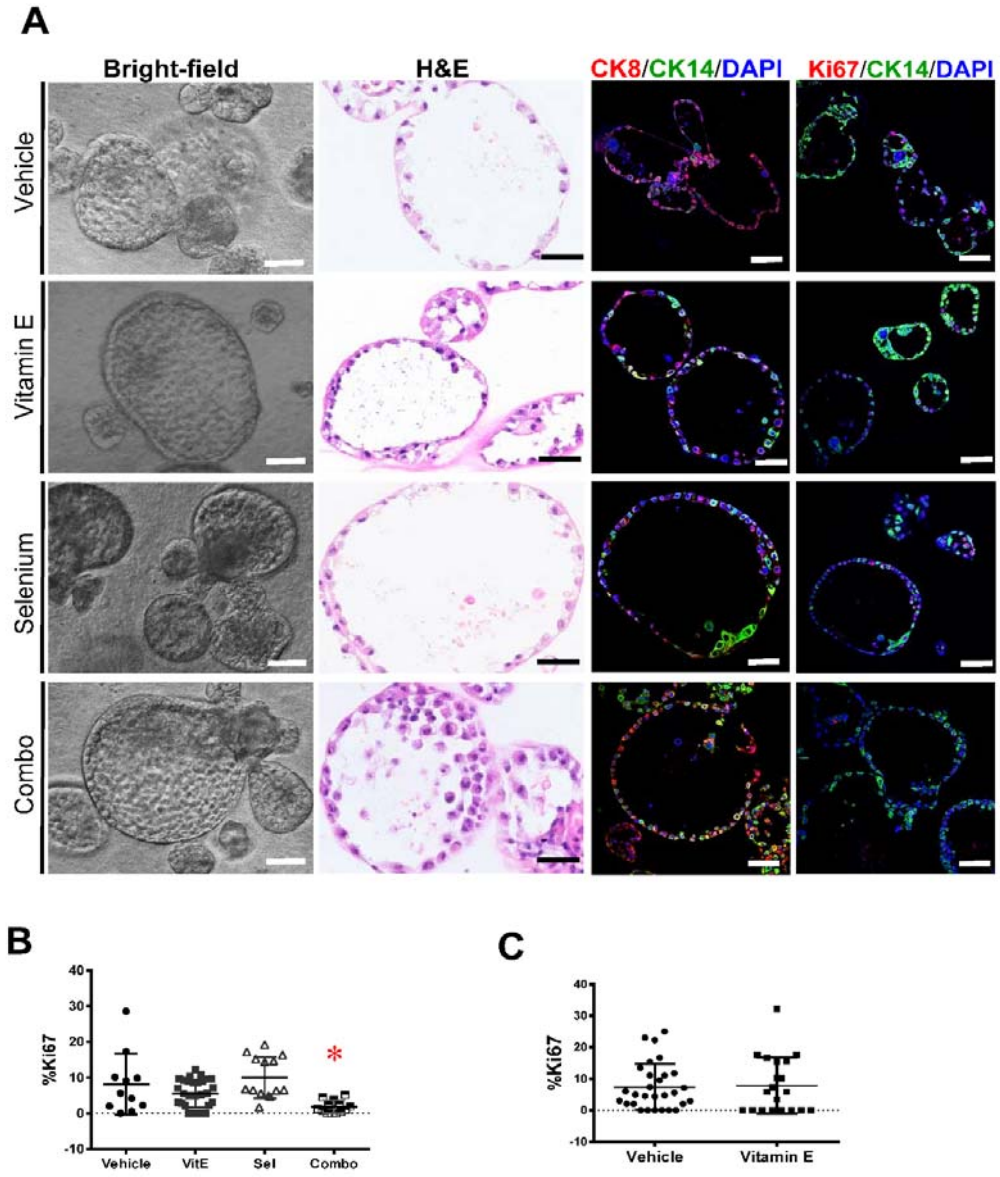

Figure 5. Vitamin E has no significant effect on the proliferation of healthy primary organoids. To model the SELECT trial, organoids from normal primary prostate cells of two subjects were treated with the SELECT agents. (A) Bright-field view showing several phenotypes of organoids from one of the subjects. The H&E stain shows the formation of large organoids with hollow lumens while the immuno-staining shows that these organoids expressed both luminal CK8 and basal CK14 epithelial markers. (B) Ki67 quantification showed no significant impact for vitamin E or selenium but their combination decreased organoid proliferation in the first subject. (C) Quantification of Ki67 in organoids from the second subject showed no significant difference in proliferation between vehicle and vitamin E treatments. Each data point represents a single field of view. Scale bars represent 100μm for Brightfield and IF images and 50μm H&E. Asterisks represent statistical significance (One way ANOVA with Tukey's correction for multiple comparisons or a two tailed t-test). * $p \leq 0.05$; error bars represent SD.

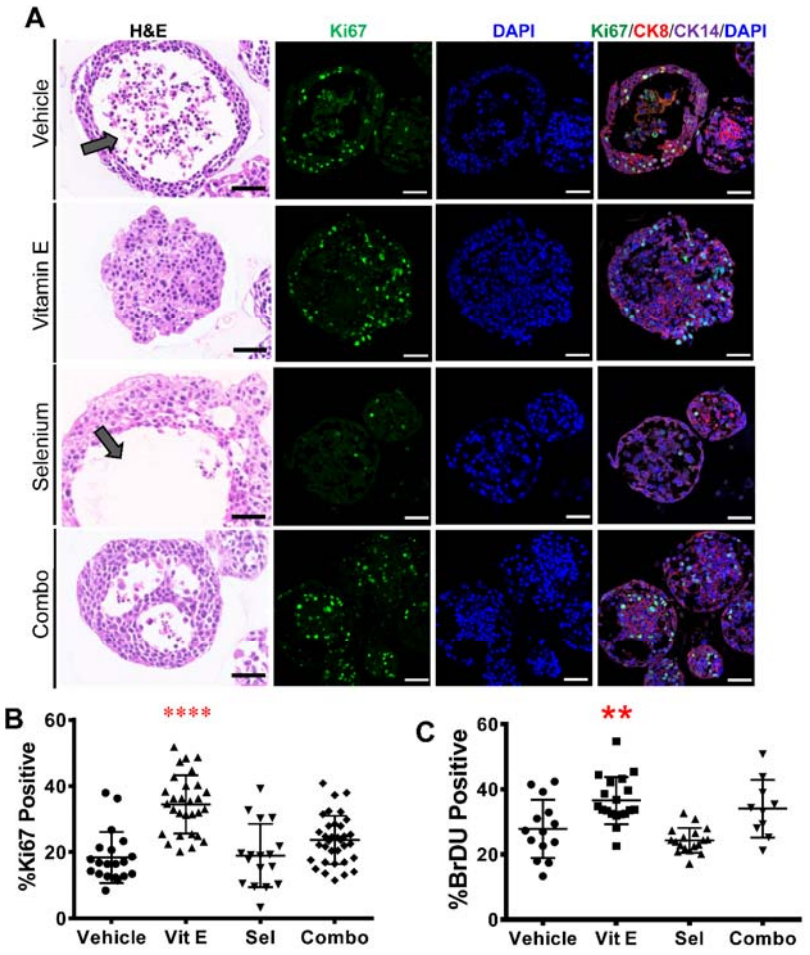

Figure 6. Vitamin E alone drives proliferation in organoids from the non-tumorigenic prostate cell line, RWPE-1, recapitulating the SELECT trial. Antioxidant-treated RWPE-1 organoids were sectioned and stained with H&E or anti-CK8, CK14 and Ki67 antibodies (A) H&E staining showed that organoids treated with vehicle or selenium had mostly hollow lumens (arrows) compared to the filled morphology in vitamin E treated organoids. The confocal images show basal and luminal staining and increased Ki67 detection in vitamin E treated organoids (B) Quantification of the percentage of Ki-67 positive cells showed a highly significant increase in proliferation in organoids treated with vitamin E alone. (C) Quantification of the percentage of BrDU incorporating cells in RWPE-1 organoids after two weeks of culture showed that vitamin E alone significantly increased the number of dividing cells. Each data point represents a single field of view. Scale bars represent 50 μm. Asterisks represent statistical significance (One way ANOVA with Tukey's correction for multiple comparisons). **$p \leq 0.005$, ****$p \leq 0.0001$; error bars represent SD.

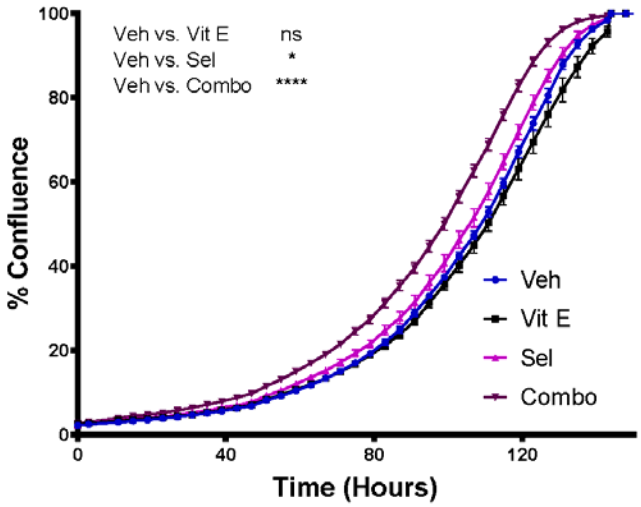

Figure 7. RWPE-1 cells in 2D do not recapitulate the results of the SELECT trial. Percent confluence over time of RWPE-1 cells with SELECT supplement treatment. Asterisks represent statistical significance (Two way ANOVA with Tukey's correction for multiple comparisons). *$p \leq 0.05$, ****$p \leq 0.0001$; error bars represent SD ($n = 6 – 9$).

Proliferation in the premalignant organoids is independent of antioxidant structure or mechanism of action

To test the effect of other antioxidants, we treated RWPE-1 organoids with a different vitamin E isomer, γ-Tocopherol or NAC (Fig. 8). Organoids treated with γ-Tocopherol alone or in combination with selenium had a filled lumen morphology (Fig 8A). Further, γ-Tocopherol increased proliferation to 34% Ki67 positivity compared to vehicle at 19% (Fig. 8C). The combination of γ-Tocopherol and selenium also had a higher proliferation rate compared to

vehicle at 29% (Fig. 8C). Therefore, unlike α-Tocopherol (Fig. 6B), the addition of selenium did not greatly attenuate the effect of γ-Tocopherol (Fig. 8C).

Whereas vitamin E isomers are lipophilic antioxidants that prevent lipid peroxidation, NAC is a precursor in the synthesis of the intracellular antioxidant glutathione.[520,521] NAC-treated organoids had a dose-dependent proliferation increase and filled lumens (Figs. 8B & D). Further, to determine whether the SELECT supplements affect the levels of ROS in RWPE-1 organoids, we quantified fluorescence in treated organoids stained with mitochondrial CellROX probes. The SELECT supplements significantly lowered mitochondrial ROS (Figs. 9A & B).

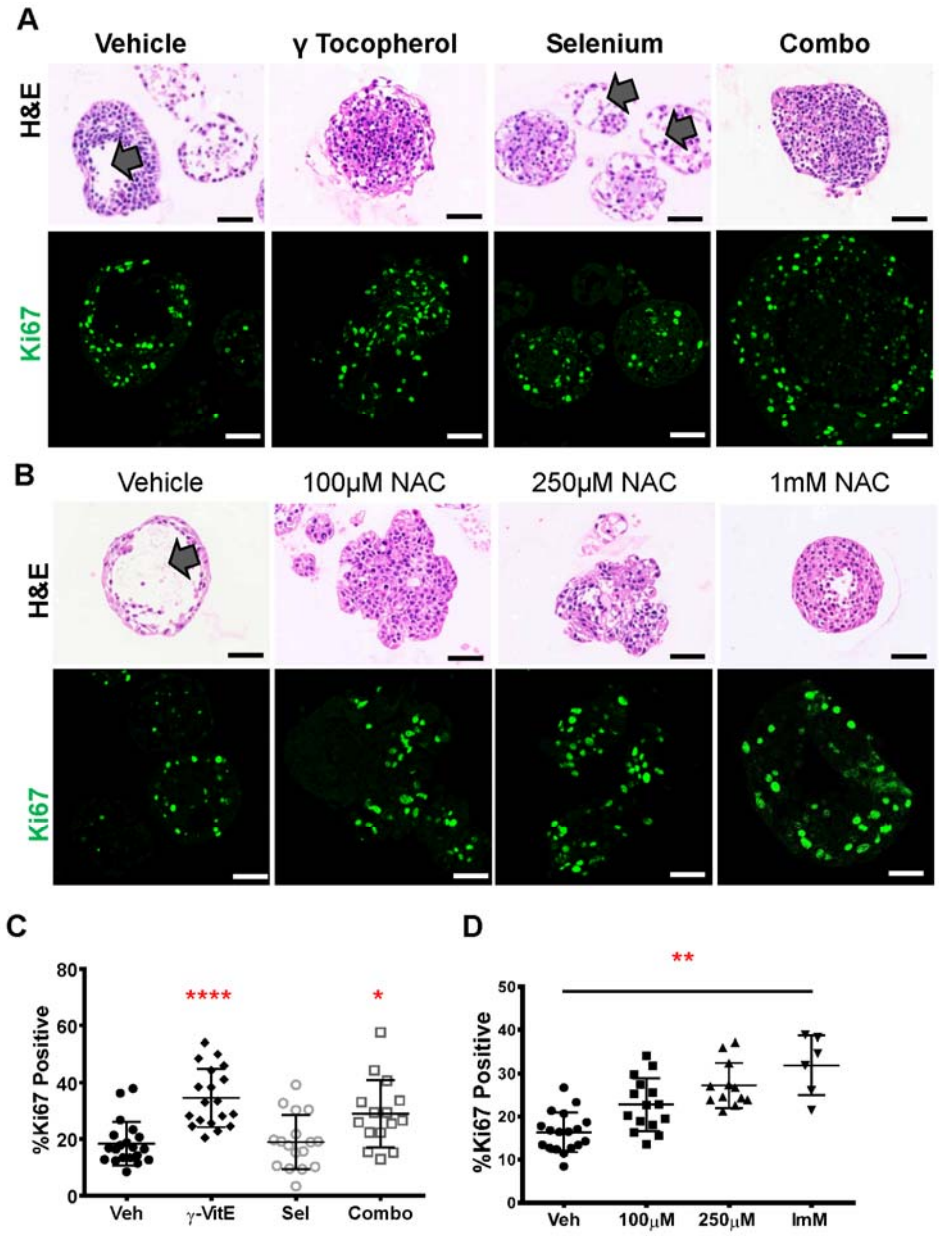

Figure 8. The proliferative phenotype in RWPE-1 organoids is independent of the Vitamin E isomer or antioxidant structure. (A) H&E staining showing hollow lumens (arrows) in vehicle and selenium and filled lumens for γ-Tocopherol or its combination with selenium. The confocal images show Ki67 immunostaining on sections of RWPE-1 organoids treated with the vehicle, γ-Tocopherol, its combination with selenium or selenium alone (B) H&E images showing hollow and filled morphology in vehicle and NAC treated organoids respectively and Ki67 immunostaining on sections of RWPE-1 organoids treated with vehicle or increasing concentrations of NAC (C) Quantification of Ki-67 positive cells from (A) showed a significant increase in the proliferation of organoids treated with γ-Tocopherol or its combination with selenium but not selenium alone. (D) Quantification of Ki-67 positive cells from (B) showed a dose-dependent increase in proliferation in organoids treated with NAC. Each data point represents a single field of view. Scale bars represent 50 μm. Asterisks represent statistical significance (One way ANOVA with Tukey's correction for multiple comparisons). *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$; error bars represent SD.

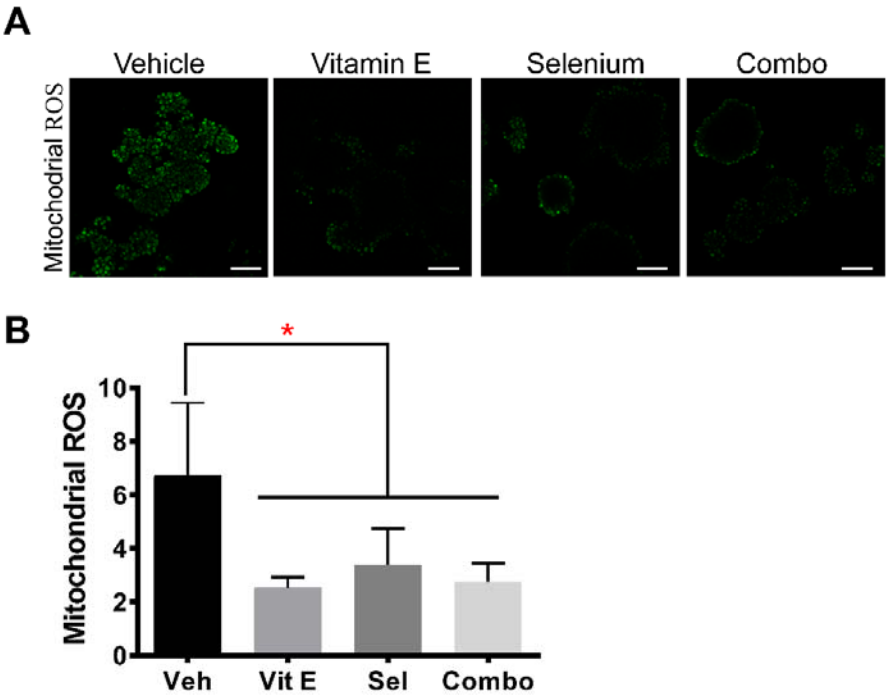

Figure 9. All SELECT agents significantly decreased mitochondrial ROS. To detect mitochondrial ROS in RWPE-1 organoids, we used a redox probe (CellROX Green) that is non-fluorescent in its reduced state but which fluoresces proportionately upon oxidation. (A) RWPE-1 organoids treated with the SELECT agents were stained with the probe and imaged by confocal microscopy. (B) Quantification of the mean fluorescence intensity of the images in (A) showed a significant reduction in mitochondrial ROS by all the SELECT agents. Scale bars represent 100 µm. Asterisks represent statistical significance (One way ANOVA with Tukey's correction for multiple comparisons). *$p \leq 0.0001$, error bars represent SD.

Microarray analysis revealed opposing effects of vitamin E and selenium on cell proliferation in the premalignant organoids

To gain further insight into the effects of SELECT supplements on RWPE-1 organoids, we performed gene expression profiling using microarrays followed by gene-set enrichment analysis (GSEA).[514] Vitamin E upregulated cancer and cell cycle related gene sets that were suppressed by selenium and the combination treatments (Figs. 10 – 12). The leading-edge subsets describe

the genes that contribute most to a gene set's enrichment score and thus have the highest correlation with the phenotype of interest.

The leading-edge analysis showed an upregulation of cell cycle and genome replication genes including cyclins and mini-chromosome maintenance proteins (MCM) by vitamin E but downregulated by selenium and the combination treatments (Table 2). The GSEA results were visualized with the Cytoscape Enrichment Map plug-in, which groups significant gene sets into functional networks based on annotation similarity and gene overlap.[515,516] The key network affected by all the treatments was cell proliferation which was upregulated by vitamin E (Figs. 13A & B), but suppressed by selenium (Figs. 13C & D) and the combination treatment (Figs. 14A & B).

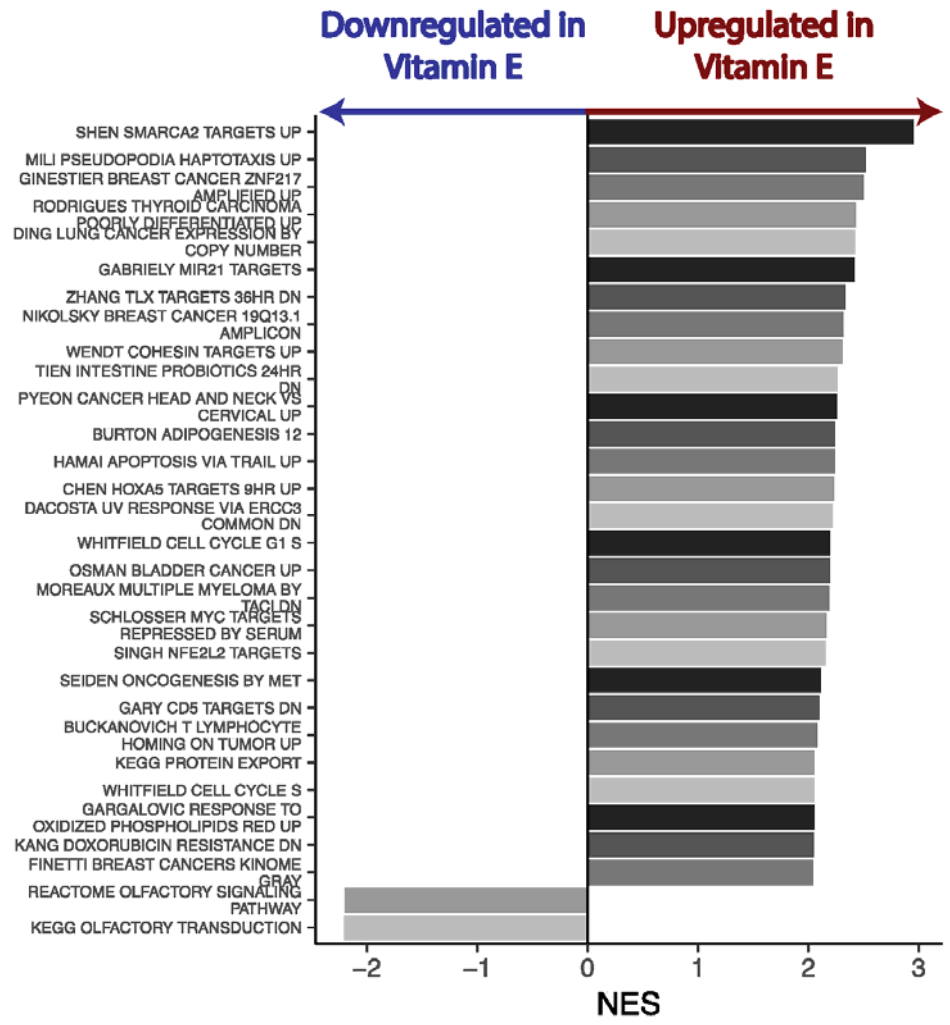

Figure 10. Top 30 gene sets that are significantly enriched by vitamin E identified by gene-set enrichment analysis (GSEA) of differentially expressed genes (p value ≤ 0.005)

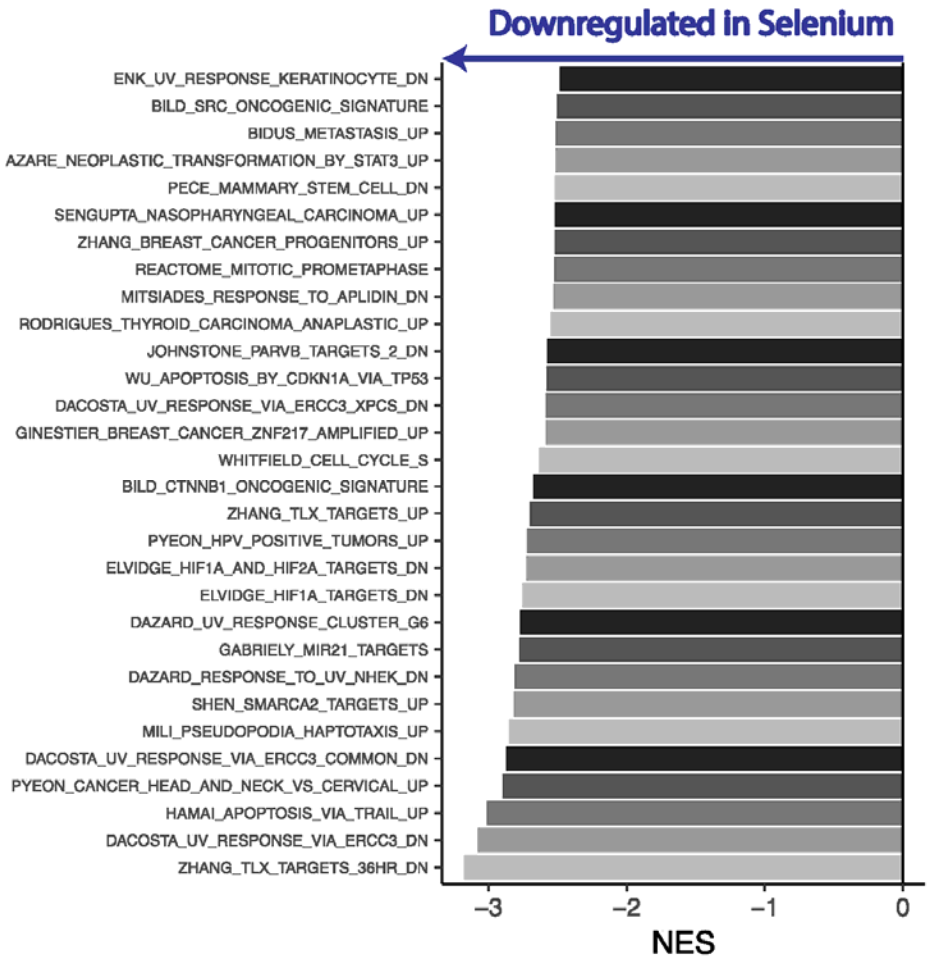

Figure 11. Top 30 gene sets that are significantly enriched by selenium identified by gene set enrichment analysis (GSEA) of differentially expressed genes (p value ≤ 0.005).

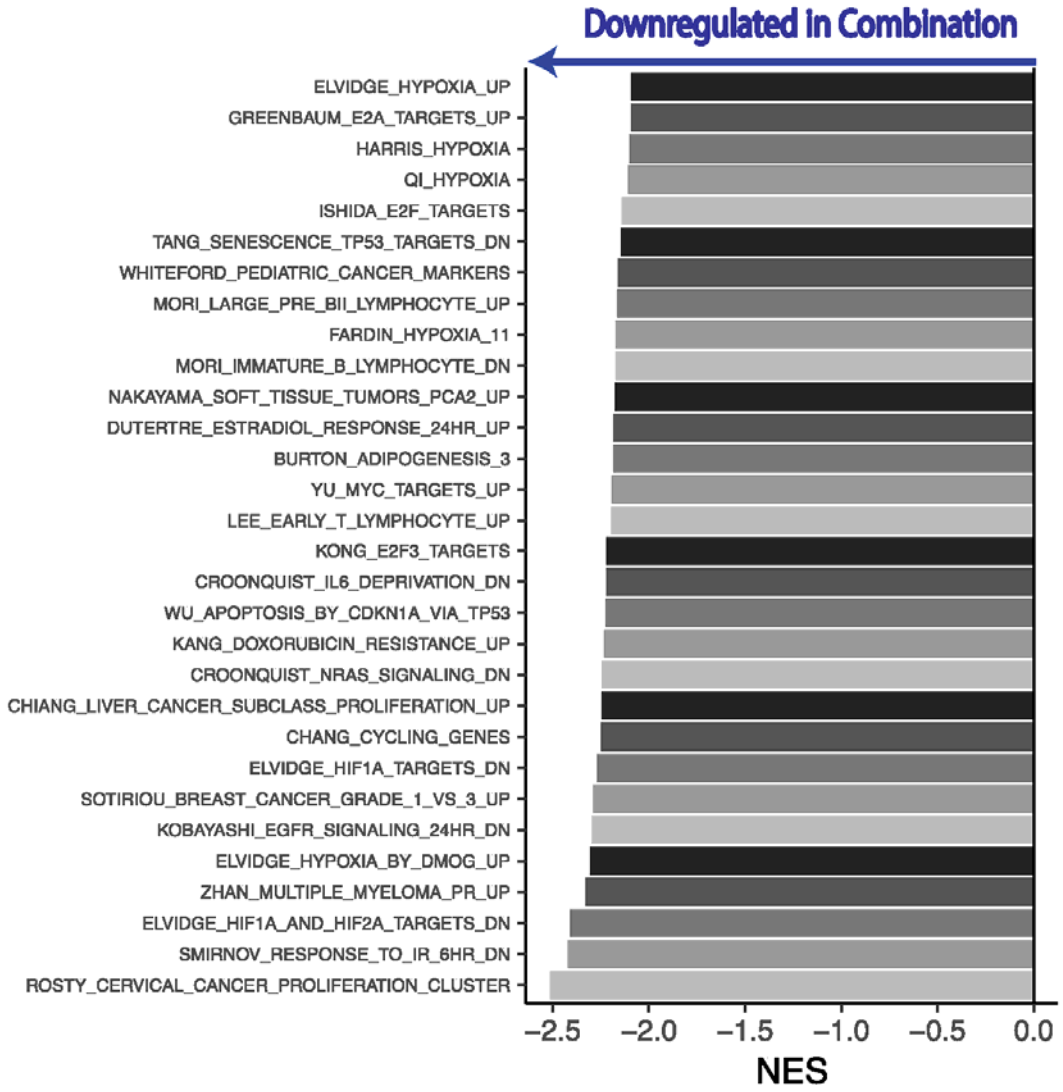

Figure 12. Top 30 gene sets that are significantly enriched by vitamin E and selenium combined identified by gene set enrichment analysis (GSEA) of differentially expressed genes (p value ≤ 0.005).

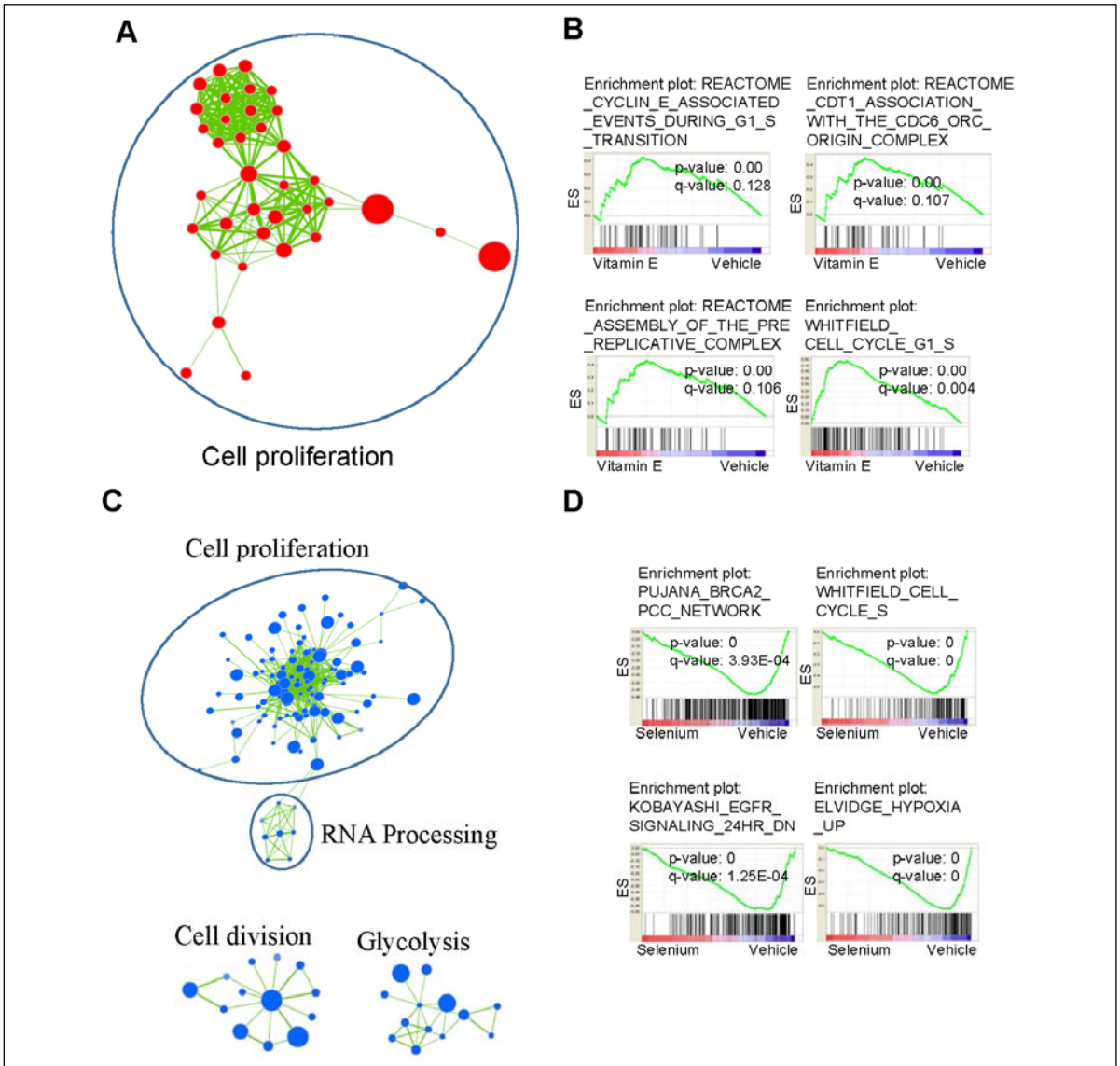

Figure 13. In RWPE-1 organoids, Vitamin E upregulates a cell proliferation gene network which is suppressed by Selenium. We used Cytoscape to cluster together functionally related gene sets found to be significantly enriched after querying our microarray data with MSigDB's C2 curated gene sets using GSEA. We show networks containing ≥ 5 gene sets (False Discovery Rate q value < 0.25). Node size corresponds to gene set size. Red circles represent up-regulation and blue circles represent down-regulation of the gene set. Colour intensity represents significance by enrichment p value. Line thickness connecting the gene set nodes represents the degree of gene overlap between the two sets. (A)Vitamin E upregulated a gene set network associated with cell proliferation (B) Example GSEA enrichment plots for selected gene sets from the vitamin E network. The Enrichment Score (ES; y-axis) reflects the degree to which a gene set was upregulated (cumulative positive score) or down-regulated (cumulative negative score) in the treatment group. Each vertical line in the 'bar code' represents a single gene in a gene set. Hue designates the direction in which the genes are altered (red = enriched in vitamin E, blue = depleted in Vitamin E). Nominal p value (statistical significance of the enrichment) and the FDR are indicated. (C) Selenium downregulated cell proliferation, glycosis gene networks among others (D) Example GSEA enrichment plots for selected gene sets from the selenium affected networks.

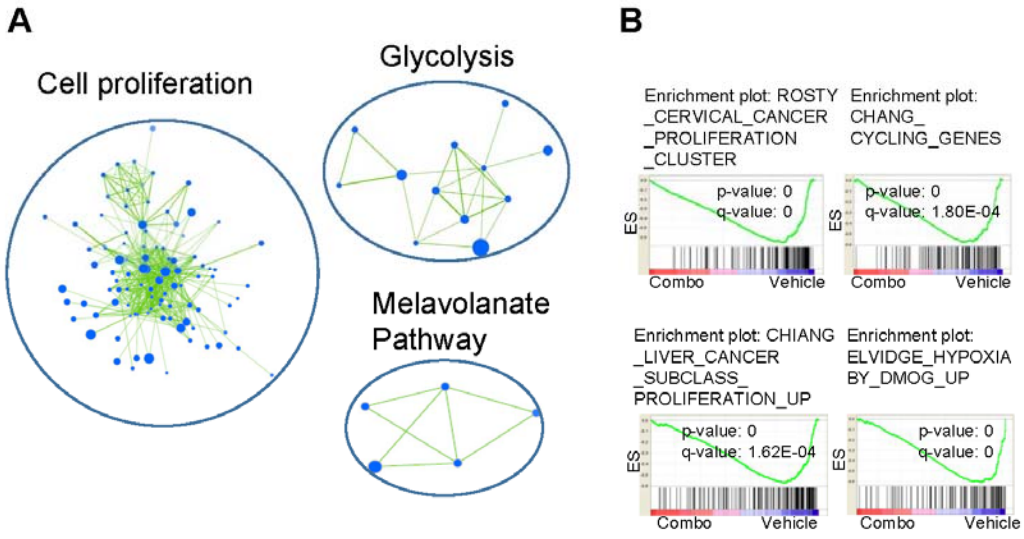

Figure 14. The combination of Vitamin E and Selenium suppresses cell proliferation, glycolysis and lipid metabolism pathways in RWPE-1 organoids. (A) Cytoscape visualization of gene sets enriched in RWPE-1 organoids treated with a combination of vitamin E and selenium. We show network with ≥ 5 functionally related gene sets (False Discovery Rate q value < 0.25). (B) Example GSEA enrichment plots for selected gene sets making up the networks shown in (A). Nominal p value (statistical significance of the enrichment) and the FDR are indicated.

Suppression of glucose uptake leads to a drop in ATP generation in detached premalignant cells

From the gene expression analysis, we observed a downregulation of glucose transporters and glycolytic enzymes to varying degrees among the treatments (Fig. 15A). Several integrins which mediate ECM cell attachment were also downregulated pointing to loss of matrix attachment (Fig. 15A). Studies in mammary organoids have demonstrated differences in the glycolytic rates between the outer ECM attached and the inner detached cells.[522] We therefore tested whether suppression of the glycolysis pathway was associated with differences in glucose absorption and glycolysis. We used adherent and nonadherent cells on poly-HEMA coated plates to mimic the

attached matrix proximal cells (outer) and detached matrix distal (inner) organoid cells respectively.

The non-ionic nature of poly-HEMA prevents ECM deposition on tissue culture plates, poly-HEMA coated tissue culture plates prevent cellular attachment and spreading.[523] *In vitro* growth in soft agar, the gold standard for measuring anchorage-independent growth, correlates with growth of human epithelial cancer cells on poly-HEMA coated plates and tumorigenicity.[524,525] Therefore, growth of human epithelial cancer cells on poly-HEMA is also an indicator of tumorigenecity.[525] As such, poly-HEMA adhesion deprived cell cultures provide a model for studying the regulation of anchorage-independent cell survival and growth especially for studies that are difficult to perform on cells embedded in soft agar or matrigel like in organoids.[526]

With this approach, several studies have elucidated alterations in various cellular metabolic pathways under matrix nonadherent conditions.[522,527-529] We found that, adhesion deprived RWPE-1 cells significantly reduced glucose absorption, and this was not rescued by the addition of antioxidants (Fig. 15B). Consequently, detached RWPE-1 cells had significantly lower levels of basal and maximal ECAR, a measure of lactate production from glycolysis, compared to attached cells which was not rescued by the addition of vitamin E (Figs. 16A – C).

Next, we measured ATP levels under the same conditions to determine the effect of reduced glycolysis and antioxidants on cell energetics. The detached cells had significantly lower ATP levels compared to attached cells (Fig. 16D). Though vitamin E did not rescue glycolysis, it did rescue the diminished ATP levels in detached cells (Fig. 16D) consistent with findings in

mammary cells.[522] One caveat of the poly-HEMA suspension assay is that it can lead to death by anoikis. To minimize this effect, all assays were conducted within 24 hours.

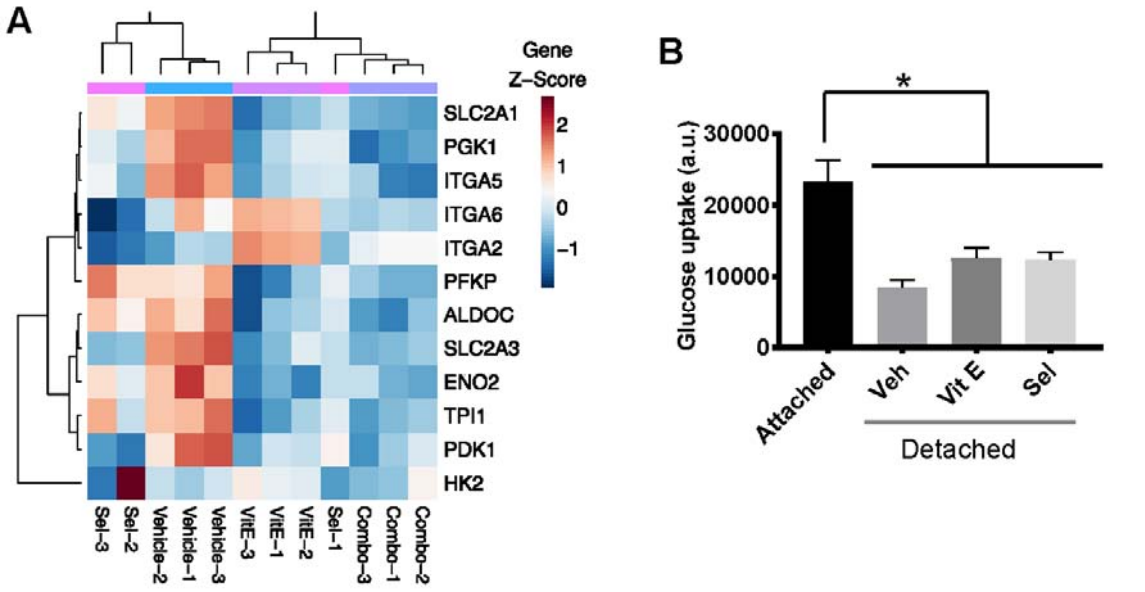

Figure 15. Antioxidant treated organoids display ECM detachment and downregulation of glucose transporters lowering glucose uptake (A) Heat map of differentially expressed genes in the glycolysis and cell attachment pathways identified to be affected by the gene set analysis showed a downregulation of both pathways to varying degrees by antioxidant treatment (B) Measurement of glucose uptake in adherent or non-adherent, (poly-HEMA coated plates), RWPE-1 cells showed a significant decrease in the detached cells that was not rescued by the antioxidants after 24h.

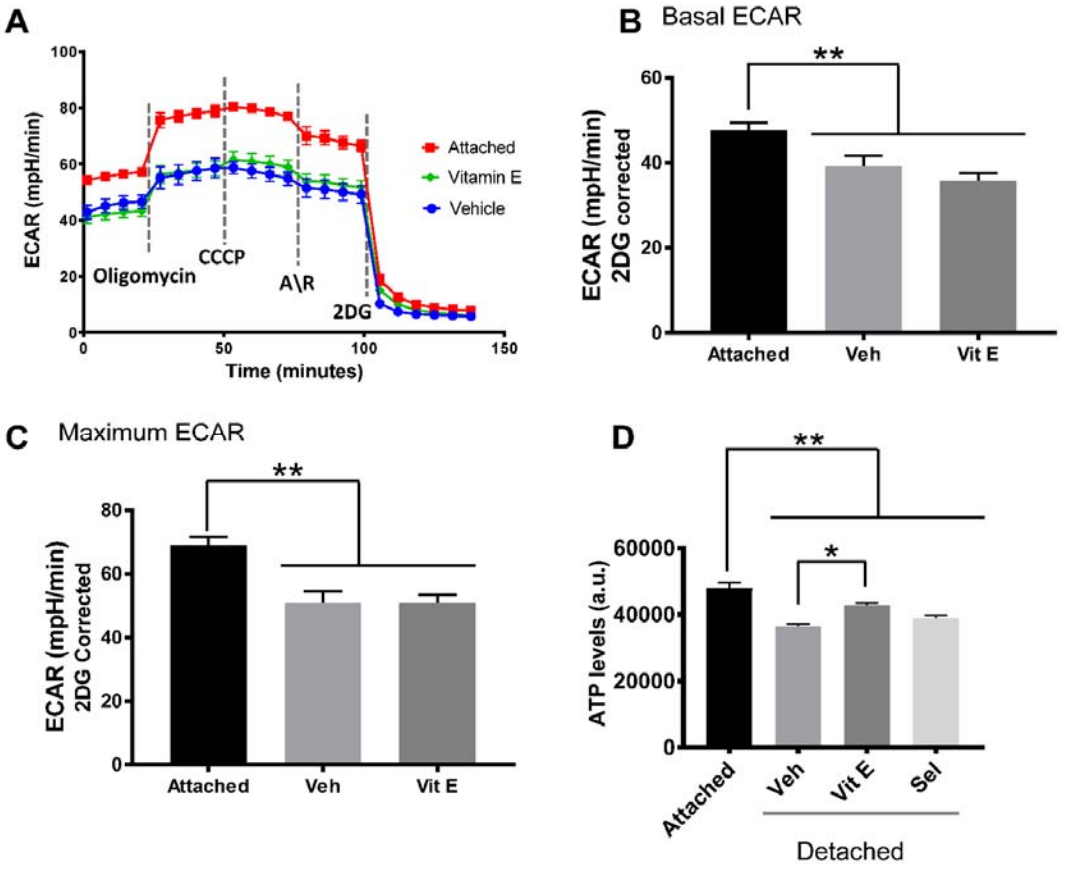

Figure 16. Vitamin E promotes cell survival through a metabolic rescue of ECM detached cells. (A) ECAR analysis of RWPE-1 cells grown in adherent and non-adherent conditions and treated with the SELECT supplements for 24h, n = 15. (B) Basal ECAR was calculated by subtracting non-glycolytic ECAR from the basal ECAR measurements vs vehicle. (C) Maximal ECAR was calculated by subtracting non-glycolytic ECAR from the ECAR measurements after the addition of oligomycin. (D) ATP levels measured in 24h RWPE-1 cell cultures in adherent or non-adherent plates showed a significant ATP rescue by Vitamin E treatment, ($n \geq 3$). Asterisks represent statistical significance (One way ANOVA with Tukey's correction for multiple comparisons). *$p \leq 0.001$, **$p \leq 0.0001$; error bars represent SD. A.U. means arbitrary units.

Vitamin E increases the survival of detached premalignant cells by stimulating fatty acid oxidation

Next, we sought to determine whether this ATP rescue despite the decreased glycolytic flux was through increased oxidative phosphorylation, the other major pathway for energy generation.

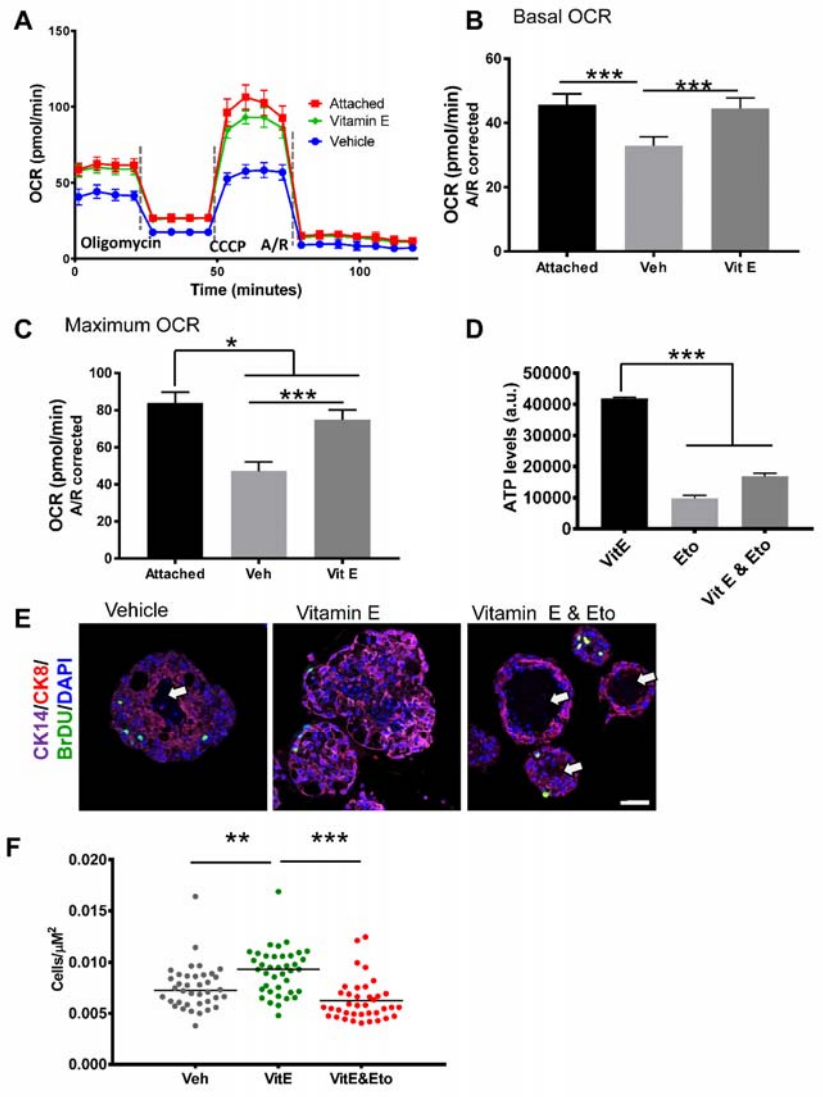

Figure 17. Vitamin E restores ATP levels in detached cells by stimulating fatty acid oxidation leading to organoid luminal filling. (A) OCAR analysis of RWPE-1 cells grown in adherent and non-adherent conditions and treated with the SELECT supplements for 24h, n = 15. (B) Basal OCAR was calculated by subtracting non-mitochondrial OCAR from the basal OCAR measurements. (C) Maximal OCAR was calculated by subtracting non-mitochondrial OCAR from the OCAR measurements after the injection of CCCP. (D) Detached RWPE-1 cells were treated with vehicle, vitamin E or vitamin E and an FAO inhibitor, Etomoxir (Eto, 25μM) for 24h; ATP measurement showed that FAO inhibition abrogated the ATP rescue by vitamin E. (E) Immunostained sections of RWPE-1 organoids from (D), showed that the vitamin E treated organoids had filled lumens while those co-treated with Vitamin E and Etomoxir or vehicle had hollow lumens (arrows). (F) Cell density of organoids from (E) measured by dividing the number of total cells per organoid by its area showed that the vitamin E organoids had the highest cell densities while those co-treated with vitamin E and Etomoxir had the lowest cell densities. Scale bars represent 100 μm. Asterisks represent statistical significance (One way ANOVA with Tukey's correction for multiple comparisons). **$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.0001$, error bars represent SD.

To this end, we measured changes in oxygen consumption rate (OCR), which is linked to mitochondrial oxidative phosphorylation. Whereas vehicle-treated detached cells had low basal and maximum OCR, those treated with vitamin E had significantly higher OCR levels that were comparable to the attached cells (Figs. 17A – C). When glucose is depleted, cells can derive energy from fats through fatty acid oxidation (FAO). Since vitamin E did not rescue glucose uptake, we tested whether it might stimulate FAO. ATP levels were measured in non-adhering, antioxidant treated RWPE-1 cells with or without Etomoxir, an FAO inhibitor. FAO inhibition abrogated the ATP rescue by antioxidants (Fig. 17D). To move these findings to a more physiologically relevant model, we tested the effect of FAO inhibition in vitamin E-treated RWPE-1 organoids.

FAO inhibition in vitamin E treated organoids selectively killed the inner, ECM detached cells, reverting the filled lumen morphology back to a normal hollow morphology (Fig. 17E). Consequently, vitamin E treated organoids had the highest cell densities while those co-treated with etomoxir had the lowest cell densities pointing to increased cell survival (Fig. 17F).

CHAPTER 4: DISCUSSION

The role of antioxidants in cancer chemoprevention has been controversial. The surprising findings from the SELECT trial showing an increased risk of prostate cancer with vitamin E supplementation have been the subject of much discussion.[496] The discordance between the *in vitro* studies that informed SELECT's design and the trial's outcome is partly due to the predominant use of conventional 2D tissue culture which bears little resemblance to the *in vivo* environment. For mechanical support, the cell monolayer in 2D cultures adheres to a flat surface typically glass or polystyrene, giving the cells an abnormally flattened and stretched morphology, which impacts cellular behavior, growth and function.[505,530]

In addition, 2D cell epithelial cultures lack native tissue architecture due to loss of apical-basal polarity and lack of the natural three-dimensional (3D) environment in native tissue where cells are surrounded by other cells and the extracellular matrix (ECM).[505,531] Furthermore, 2D cultures also fail to model pertinent aspects of human tumors. Unlike in human tumors, 2D monolayers have equal access to nutrients and growth factors in the culture media resulting in homogenous cell proliferation.[505] In contrast, epithelial cells embedded in matrices like collagen or reconstituted ECM (matrigel) aggregate in three dimensions (3D) forming native like cell-cell and cell-matrix interactions bridging the gap between artificial 2D *in vitro* and *in vivo* conditions.[531]

Moreover, nutrient, growth factor and oxygen gradients in 3D cultures gives rise to heterogeneous cell populations of proliferating, quiescent, apoptotic or necrotic and hypoxic cells similar to poorly vascularized tumors.[505] In addition to providing physical support and

serving as a scaffold for tissue organization, ECM attachment also provides biochemical and biomechanical cues necessary for anchorage-dependent cell growth, proliferation, migration and differentiation.[532] During normal epithelial glandular development, ECM–cell interactions align the mitotic spindle so that cells divide parallel and not perpendicular to the epithelial plane, and balance proliferation and cell death for tissue homeostasis and lumen morphogenesis.[533]

The normal prostate gland is a psuedostratified polarized epithelium made of basal cells, rare neuroendocrine cells and luminal cells, which secrete prostatic fluid in to a central lumen (Fig. 2).[534] In 3D culture, prostate epithelial cells induce apical-basal polarity generating lumen-containing acini resembling *in vivo* prostate gland architecture with restored tissue-specific prostatic fluid secretion hence, they are termed organoids.[535] Tissue architecture is said to be critical for epithelial tissue function.[536] Indeed, the malignant transformation of the prostate epithelium is marked by the gradual loss of cell adhesion and glandular architecture.[537-539] Therefore, 3D cultures lend themselves well to studies of both gland morphogenesis and effects of tissue architecture disruption in carcinogenesis.

We showed that premalignant but not benign or malignant prostate epithelial cells grown as 3D organoids respond to antioxidant treatment in a manner that recapitulates the findings of the SELECT trial. The supplements decreased proliferation and increased cell death in malignant LNCaP organoids, consistent with reports that show antioxidant efficacy in established cancer cell lines.[399,400] The LNCaP organoid data therefore support the concept that moderate levels of ROS damage DNA leading to mutations that can aggravate cancer.[540] In contrast, the agents with the exception of selenium significantly increased the proliferation of premalignant RWPE-1 cell organoids.

As cells at different stages of tumorigenesis experience different levels of ROS, it is reasonable to expect the antioxidant effect to be dependent on a cell's position in the tumor progression spectrum. In fact, the SELECT supplements did not affect the proliferation of benign organoids derived from primary prostate epithelial cells. These results are consistent with SELECT where just a fraction more of the men on vitamin E developed PCa compared to those on the placebo.[496] We suggest that these individuals might have harbored initiated, pre-malignant cells with molecular aberrations that were pushed in to malignancy by vitamin E.

In support of this hypothesis, Martinez *et al.*, demonstrated that the antioxidant NAC causes prostatic epithelial hyperplasia in mice with prostate specific deletion of the Nkx3.1 tumor suppressor but not in wild-type mice.[541] In addition, polymorphisms in NKX3.1 were found to modulate PCa risk in men on the interventional arms of the SELECT trial.[542] This points to the importance of the underlying genetic background of prostate cells in modifying the response to antioxidant supplementation. However, the methods used in SELECT's inclusion criteria, a PSA count and a non-suspicious DRE, could not have ruled out the existence of molecular aberrations.

Our benign organoids had proper glandular structure with well formed lumens a morphology that was not affected by vitamin E and/or selenium treatment.[543] As organoids are avascular, this allowed us to mimic the *in vivo* spatial constraints in cells in often poorly vascularized solid tumor cells. Similar to tumors, cells at the center of large organoids are under various stresses including loss of ECM attachment and limits in the diffusion of nutrients and oxygen.[544] In benign organoids, these cells die off since nutrient uptake depends on the ECM attachment status and growth factor signaling (Fig. 18).[139]

In contrast, the malignant LNCaP cells formed organoids without lumens (filled morphology) a phenotype consistent with the acquisition of anchorage-independent survival and loss of glandular differentiation. On the other hand, the vehicle treated premalignant RWPE-1 organoids had more differentiated acini structures and predominantly hollow lumens. When treated with vitamin E but not selenium however, the premalignant organoids developed a predominantly filled lumen morphology indicating increased cell survival in a low matrix environment. This further demonstrates that the premalignant RWPE-1 organoids best recapitulated results of the SELECT trial.

Microarray analysis of RNA extracted from vitamin E treated RWPE-1 organoids displayed significant downregulation of several integrins. Because integrins mediate ECM-cell attachments these results confirmed the loss of matrix attachment. The microarray data also revealed decreased expression of glucose transporters 1 and 3 (GLUT 1 and 3) and several glycolytic enzymes in the vitamin E treated RWPE-1 organoids. These results imply dysregulation of glucose metabolism in spite of increased cell growth and survival in these organoids. In mammary organoids, loss of matrix attachment rapidly induced autophagy a lysosomal degradation pathway of proteins and organelles that promotes cell survival under metabolic stress.[545,546]

In MCF-10A breast acini, measurement of native NADPH fluorescence as a metabolic read out showed that only the inner matrix deprived cells but not the outer ECM attached cells were metabolically compromised.[522] These findings confirm that loss of matrix attachment (LOA) jeopardizes cellular metabolism. Indeed, when cultured in non-adherent conditions to mimic the matrix deprived inner organoid cells, RWPE-1 prostate benign epithelial cells had significantly

reduced ATP levels and glucose uptake a phenomenal also observed in MCF-10A benign epithelial cells.[522] Altered metabolism after cell detachment has been attributed to the loss of integrin activation of the PI3K/AKT pathway, a crucial regulator of glucose and glutamine uptake and metabolism (Fig. 19).[522]

ECM-integrin contact leads to the recruitment of adopter proteins like talin and paxillin as well as signaling molecules like focal adhesion kinase (Fak) and small GTPases to form large macromolecular structures termed focal adhesions connecting the ECM and the actin cytoskeleton.[547] Autophosphorylation of FAK (Y397) downstream of integrin signaling activates its kinase function leading to the activation of the SRC/MAPK and PI3K/AKT pathways, which are crucial for progression through the G1/S checkpoint, cell survival and proliferation.[548,549] Cross talk between integrins and receptor protein tyrosine kinases (RPTKs) also activates the PI3K/AKT pathway downstream of the epidermal growth factor receptor (EGFR).[550] In contrast, matrix deprived cells in the vehicle treated malignant LNCaP organoids continued to survive and proliferate leading to the filled lumen morphology.

The accumulation of multiple alterations allows cancer cells to circumvent extracellular regulation enabling them to uptake nutrients constitutively.[139] In contrast, the PI3K pathway is important in normal epithelial cells for survival, and its inactivation by loss of ECM attachment leads to a form of cell death termed anoikis which is Greek for "homelessness."[523] *In vivo*, anoikis prevents re-adhesion of detached cells to matrices in the wrong location and their abnormal growth.[551] Autophagy activation in these cells is thought to be short-term metabolic safeguard against anoikis that presumably allows them to survive in case they are able to reattach

to the matrix.[552] Whereas prolonged autophagy can lead to cell death, a mechanism used for luminal clearance in normal mammary acini, short-term, autophagy increases cell viability.[553]

As well as diminished glucose uptake which lowered ATP generation, detached benign mammary epithelial cells also had elevated levels of reactive oxygen species (ROS).[522] This was shown to result from the decreased glucose flux through the Pentose Phosphate Pathway (PPP) for NADPH generation.[522] Interestingly, treatment of premalignant prostate organoids with the SELECT agents not only decreased ROS levels but also rescued ATP but not glucose uptake. Similar findings have been reported in detached mammary cells treated with NAC or trolox, a soluble form of vitamin E.[522] In the absence of glucose, cells can catabolize fatty acids for ATP generation. In Akt transformed glioblastoma, cells activate fatty acid oxidation (FAO) to survive upon glucose withdrawal.[181]

We showed that vitamin E treatment in detached RWPE-1 cells rescued the ATP deficiency in a FAO-dependent manner which increased premalignant cell survival and filled lumens in organoids (summarized in Figs. 18 & 19). Consequently, FAO inhibition decreased cell survival and enhanced organoid luminal clearance indicating that antioxidants are necessary for anchorage independent cell survival. It has been postulated that ROS inhibits FAO and hydrogen peroxide a form of ROS, has been shown to inhibit peroxisomal FAO.[554-556] Though the mechanism remains undefined, this implies that antioxidants activate FAO by eliminating ROS.

Finally in this study, just like in SELECT, selenium had more complex effects. In addition to decreasing ROS, selenium affected the expression of more genes in RWPE-1 organoids compared to vitamin E. Selenium is incorporated in to various selenoproteins with a broad range

of activities besides redox homeostasis.[421,557,558] It is possible that any protumorigenic effects of selenium's antioxidant function are counteracted by its effect on other anti-tumorigenic pathways. However, although selenium treatment induced an anti-proliferative gene signature, it did not lower the proliferation index in RWPE-1 organoids probably due to alternative mechanisms that override transcriptional regulation.

This study was limited by the difficulty of establishing cultures of pre-malignant prostate epithelial cells *in vitro* so we used the RWPE-1 cell line instead. Additionally, we did not directly measure FAO but used the more general mitochondrial respiration readout, OCR.

CONCLUSIONS

This study highlights the importance of neutralizing ROS for anchorage independent cell growth and survival implying that ROS can imperil detached cells. In support of this notion, other antioxidant-driven metabolic rescue mechanisms following loss of attachment have also been described.[559] In lung cancer spheroids, LOA upregulates glutamine reductive metabolism by cytosolic isocitrate dehydrogenase-1 (IDH1) to generate NADPH which is shuttled to inhibit mitochondrial ROS enhancing cell growth.[559]

Anchorage independence facilitates cell growth and survival in ectopic environments for example in metastasis.[560] Therefore, identifying mechanisms that enable anchorage independence in normal and premalignant cells offers clues on how this might be impeded to eradicate metastatic cancer cells.[560] Moreover, inactivating mutations and deletions in PTEN are frequent events seen in 20% primary PCa and 50% CRPC respectively.[561] These events lead to

activation of the PI3K pathway which is associated with metabolic alterations that enable anchorage-independent survival.

Our data suggest that antioxidants could be effective against malignant PCa but they promote tumorigenesis in premalignant cells. Moreover, these results show that the use of preclinical models that better mimic *in vivo* conditions and disease stage yield data that is more relevant for clinical translation. Finally, given the central role that metabolism plays in cancer initiation and progression, preclinical studies ought to include metabolic endpoints when assessing potential chemopreventive agents.

FUTURE DIRECTIONS

ROS are said to inhibit FAO, in particular, hydrogen peroxide has been shown to inhibit FAO in peroxisomes.[179,554-556] This implies that antioxidants activate FAO by eliminating ROS. However, the mechanism through which this happens remains undefined. It has been proposed that this might be through an indirect mechanism, by the inhibition of TCA cycle enzymes like mitochondrial aconitase, or directly by inhibiting FAO enzymes.[562,563] In addition, though we observed an increase in the proliferation index and in the expression of genes involved in cell proliferation in premalignant organoids treated with vitamin E, the downstream signaling pathways effecting this phenotype also remain undetermined.

For these questions, A CRISPR–Cas9-based genome-wide screen can be used to identify genes whose loss decreases FAO, cell survival and cell proliferation in low-attachment conditions. Not only would this uncover the mechanisms through which antioxidants increase proliferation, cell

survival and correct metabolic deficiencies after ECM detachment, but they might also unveil new therapeutic targets. Our study also did not determine what kind of ROS-induced cell death was responsible for luminal clearance in the vehicle treated organoids or how it might be regulated. In mammary acini, inhibition of apoptosis and autophagy merely delays cell death implicating alternative cell death mechanisms besides apoptosis and autophagic cell death.[526]

Uncovering the mechanism(s) responsible for the non-apoptotic cell death of prostate epithelial cells after ECM detachment could unveil therapeutic targets for the clearance of metastatic cells which are often in non-native matrix environments.

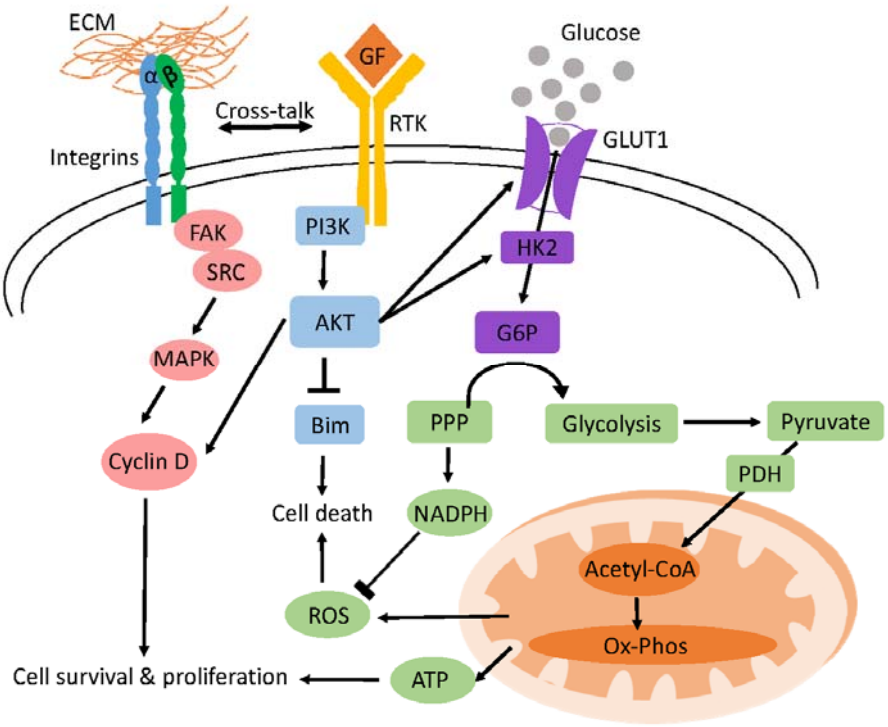

Figure 18. Model of anchorage-dependent regulation of cell survival and glucose metabolism. Adhesion of cells to the ECM activates integrins and receptor tyrosine kinase (RTK) signaling which triggers different pro-survival and proliferation pathways like AKT and MAPK. AKT inhibits BIM, a pro-apoptotic protein preventing anoikis and increases glucose uptake and glycolysis by upregulating the transcription of glucose transporters (GLUT1) and hexokinase 2 (HK2) respectively. Oxidative phosphorylation (OXPHOS) of pyruvate derived from glucose yields ATP for cellular function. Glucose shunted through the PPP pathway generates NADPH which prevents ROS induced cell death.

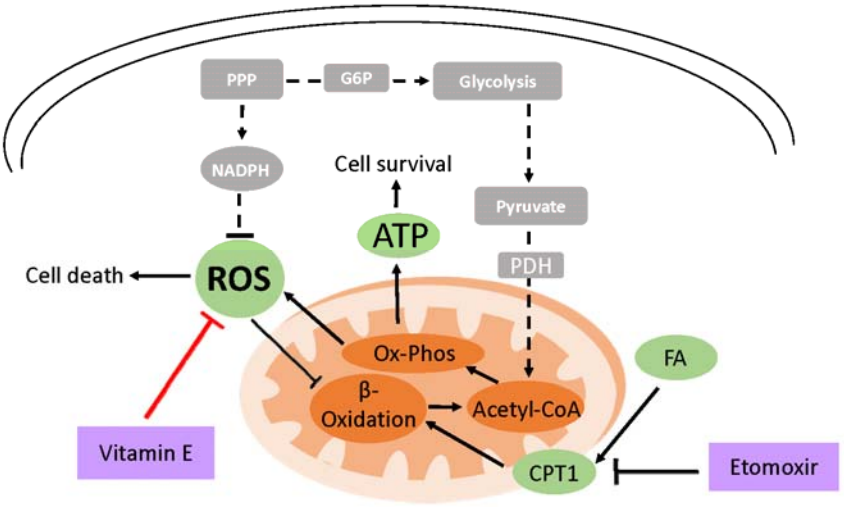

Figure 19. Model of vitamin E-mediated cell survival in ECM detached cells. Due to loss of integrin and PI3K signaling, cell detachment from the ECM results in the reduced expression of glucose transporters and low glucose uptake (shown in broken lines and grey boxes). This decreases ATP production and cell survival. Moreover, low NADPH generation through attenuated PPP flux leads to ROS accumulation which can induce cell death. ROS also inhibits fatty acid beta-oxidation (FAO) an alternative ATP generation pathway under glucose limiting conditions. However, treating detached cells with exogenous antioxidants like vitamin E neutralizes ROS which activates FAO increasing cell survival. Attenuating FAO using etomoxir which inhibits carnitine palmitoyltransferase (CPT1), FAO's rate limiting enzyme, abrogates vitamin E's ATP rescue ultimately diminishing cell survival.

Table 1. Summary of non-synonymous mutations found from the targeted sequencing of 222 cancer genes in the primary cells from the two subjects used to generate benign organoids.

Subject 1								
Gene	**Chrom**	**Exon**	**cDNA Change**	**Protein Change**	**Mutation Type**	**dbSNP142**	**OMIM Disease Association**	**ClinVar**
HNF1A	chr12	9	c.A1720G	p.S574G	missense	rs1169305	Diabetes mellitus, insulin-dependent	Pathogenic
IL7R	chr5	4	c.G412A	p.V138I	missense	rs1494555	Severe combined immunodeficiency	Pathogenic
IL7R	chr5	2	c.T197C	p.I66T	missense	rs1494558	Severe combined immunodeficiency	Pathogenic
PRSS1	chr7	2	c.A86T	p.N29I	missense	rs111033566	Pancreatitis, hereditary	Pathogenic
APOE	chr19	4	c.T388C	p.C130R	missense	rs429358	Hyperlipoproteinemia, type III /Alzheimers	Pathogenic
ATM	chr11	20	c.T2927C	p.V976A	missense	rs146145357	Unavailable	Uncertain significance
LRRK2	chr12	1	c.G149A	p.R50H	missense	rs2256408	Parkinson disease 8	Uncertain significance
LRRK2	chr12	34	c.T4939A	p.S1647T	missense	rs11564148	Parkinson disease 8	Uncertain significance
LRRK2	chr12	49	c.T7190C	p.M2397T	missense	rs3761863	Unavailable	Uncertain significance
NSD1	chr5	5	c.G1811T	p.R604L	missense	rs61744451	Unavailable	Uncertain significance

Subject 2								
Gene	**Chrom**	**Exon**	**cDNA Change**	**Protein Change**	**Mutation Type**	**dbSNP142**	**OMIM Disease Association**	**ClinVar**
PRSS1	chr7	2	c.A161G	p.N54S	missense	rs144422014	Pancreatitis, hereditary	Pathogenic
PRSS1	chr7	2	c.C47T	p.A16V	missense	rs202003805	Pancreatitis, hereditary	Pathogenic
HNF1A	chr12	9	c.A1720G	p.S574G	missense	rs1169305	Diabetes mellitus, insulin-dependent, 20	Pathogenic
PRSS1	chr7	2	c.A86T	p.N29I	missense	rs111033566	Pancreatitis, hereditary	Pathogenic

LRRK2	chr12	1	c.G149A	p.R50H	missense	rs2256408	Unavailable	Uncertain significance

Table 2. Selected leading edge genes from a sample of gene sets significantly enriched in the SELECT supplement treated premalignant organoids

Selected Leading Edge Genes Up-regulated by vitamin E	
Gene Set	**Leading Edge Genes**
Reactome Cyclin E Associated Events During G1- S Transition	CCNH CCNE2 PSMA3 CCNA1 RB1 PSMA4 CDK7 PSME2 PSMA5 PSMB3 PSMD11 PSMD12 PSMA7 WEE1 MNAT1 RPS27A PSMA6 PSMC2 PSMA1 PSMD7 PSMA2PSMC6 PSMD14 PSMB9 SKP1 MAX PSMB7 PSMB5 PSMD6 PSMC5 PSMB1
Reactome CDT1 Association With CDC6 ORC Origin Complex	PSME4 CDC6 PSMA3 MCM8 PSMA4 ORC3 ORC5 PSME2 ORC4 PSMA5 PSMB3 PSMD11 PSMD12 PSMA7 RPS27A PSMA6 PSMC2 PSMA1 PSMD7 GMNN PSMA2 PSMC6 PSMD14 PSMB9 PSMA8 PSMB7 PSMB5 PSMD6 PSMC5 PSMB1 ORC6
Reactome Assembly Of The Pre Replicative Complex	PSME4 CDC6 PSMA3 MCM8 E2F3 PSMA4 ORC3 ORC5 PSME2 ORC4 PSMA5 MCM6 PSMB3 PSMD11 PSMD12 PSMA7 RPS27A PSMA6 PSMC2 PSMA1 PSMD7 GMNN PSMA2 PSMC6 PSMD14 PSMB9 PSMA8 PSMB7 PSMB5 PSMD6 PSMC5 PSMB1 ORC6
Whitfield Cell Cycle G1-S	SLC25A27 DNAJC3 SERPINB3 HELLS TTC14 ZRANB2 LUC7L3 ZNF141 NPAT CCNE2 FAM111B CDC6 LOC400879 RAB23 PCNAP1 PNN PMS1 ARGLU1 TIPIN SLC25A36 LNPEP NKTR ANKRD10 MDM1 INTS8 CLSPN DONSON CREBZF USP53 SEC62 SPIN3 DTL CEP57 NUP43 ACYP1 RNPC3 TOPBP1 C14ORF142 ATAD2 OSBPL6 MSH2 NASP TRA2A NEAT1 MCM6 ZNF852 FBXL20 PCNA DNAJB9 DIS3 CAPN7 WDR76 IVNS1ABP BRD7 SPIN4 CASP8AP2 MBOAT1 RNF113A SSR3 GINS3 TAF15 EIF2A GMNN
Selected Leading Edge Genes Down-regulated by selenium	
Gene Set	**Leading Edge Genes**
Whitfield Cell Cycle S	CRLS1 RAD18 KIAA1598 RMI1 UBL3 KDELC1 CALM2 SVIP MASTL NRD1 LIPH NAB1 MBD4 GPR126 NT5DC1 NFE2L2 CDC45 EIF4EBP2 TRIM45 PILRB DYNC1LI2 ZNF217 ABHD10 PHTF2 DNAJB4 CASP2 PHOSPHO2 PRIM1 MAP3K2 MAN1A2 DCAF16 BMI1 RRM2 RAD51 EXO1 ABCC5 BRCA1 CALD1 MYCBP2 DONSON FAM178A ZWINT SLC22A3 TYMS USP1 BBS2 ENOSF1 DHFR NUP160 TMCC1 EFHC1 PHTF1 INTS7 HIST1H4H ESCO2 NSUN3 STAG3L1 SRSF5 KAT2B MCM8 FANCI PHIP ANKRD18A INSIG2 CDC7 PTAR1 ATAD2 ZBED5 SLC38A2 SRSF10 DMXL2 BRIP1 NEAT1 OGT C5orf42 SLC25A27 POLA1 LYRM7 TOP2A HELLS BIVM CREBZF DNA2 CCDC14 CCDC84 GOLGA8A GOLGA8B LOC389831 ANKRD36 CHML
Elvidge Hypoxia Up	AHNAK2 DTNA CYP1B1 CSRP2 BBX ATXN1 ALDOC IGFBP3 DPYSL2 GYS1 MET BNIP3 ANKZF1 YEATS2 MXI1 NDRG1 SORL1 GBE1 JUN DSC2 FAM13A SAMD4A SLC2A1 SRD5A3 TNFAIP8 CD59 KLF7 TRA2A EGFR SFXN3 CAV1 PGK1 FAM162A SPOCK1 TMEFF1 BNIP3L SLCO4A1 INSIG2 RLF ANG CCNG2 KRT7 EGLN1 VEGFC ENO2 DAAM1 VLDLR TXNIP GJA1 HK2 KDM3A PDK1 ZMYND8 DST TMEM45A SRPX LOXL2 RBPJ ANGPTL4 PAM TGFBI ERO1L P4HA1 ZNF292 WSB1 LOX PGAP1 ITPR1 EGLN3 CA9 STC1
Dacosta UV Response via ERCC3 TTD Down	UBXN7 DNAJC2 AVL9 MSH3 ACAP2 ITCH INTS3 ARHGEF10 GPATCH8 NFIB SLC16A7 ATXN1 SKAP2 RB1CC1 ROR1 AMPH NAV3 WDR37 SON PIK3C2A TMCC1 ARAP2 SERPINB2 FAM179B KLHL20 KIAA0922 PLCE1 MPHOSPH9 VPS13B USP15 TEAD1 PHF14 PDS5B CDC42BPA PVRL3 WDHD1 MALT1 TSC22D2 DOCK9 DOCK4 DST HERC4 BTAF1 LRP6 BICD1 HAS2
Kobayashi EGFR Signaling 24HR Down	KIF2C NCAPD3 ABCE1 KIF4A CDK1 PRIM1 GPSM2 RACGAP1 POLA2 GINS1 RRM2 NAA15 CDK2 NUDT15 RAD51 EXO1 MET RAD54B DUSP6 BRCA1 VRK1 DONSON ZWINT ZC3HAV1 NEK2 TYMS USP1 MCM6 SHCBP1 NCAPG2 MAD2L1 DHFR GJB3 PRC1 KIF15 BUB1 DNAJC9 DSN1 DKK1 TPX2 CCNA2 ITGA6 DCBLD2 ECT2 STEAP1 MELK TFPI2 TMEM194A SLCO4A1 FAM111A ELL2 MKI67 DEPDC1 FANCI CEP55 NT5E STIL SPC25 SMC4 ATAD2 NOC3L KIF23 MYBL1 PNN ERCC6L ENO2 KIF14 ZWILCH DTL DUSP4 NUSAP1 NRG1 PLK4 DLGAP5 LMNB1 CCNE2 SRSF7 PBK POLA1 NDC80 NCAPG HMMR TOP2A HELLS SMC2 HMGA2 ASPM STC1
Pujana BRCA Centered Network	NFYB RAD54L MCM4 DCP2 TTF2 RBBP8 SNRPA1 UBE2C RRM1 RFC3 POLR2B RAD21 SMC1A PAICS RPIA UNG DEK PPP2R5C PRKDC HMGB2 POLE RBBP4 NAE1 LBR BUB3 PPP1CC PCNA ZNF330 AURKA MSH2 NCK1 CDK1 PSIP1 GINS1 H2AFV RB1 SUZ12 BRCA1 SSBP2 NASP SMC3 MCM6 TOPBP1 MAD2L1 DHFR XPO1 MED20 TCERG1 MAT2A CCNA2 BRCA2 TMEM194A FANCI SMC4 CDC7 MRE11A RECQL LMNB1 SKP2 DDX46 ATM SRSF11 POLA1 NDC80 HMMR TOP2A SMC2 DNA2
Selected Leading Edge Genes Down-regulated by Combination Treatment	
Gene Set	**Leading Edge Genes**
Rosty Cervical Cancer Proliferation Cluster	TACC3 DNA2 MAD2L1 ACACA KIF15 PAQR4 ATAD2 CHEK1 HELLS CA2 CDC20 MELK AURKB E2F8 EBP CENPA CCNE2 TRIP13 PCNA AURKA FANCI KIFC1 H2AFX ERCC6L TTK PTTG1 CDCA3 TK1 SHCBP1 RAD51AP1 STIL TYMS POLA2 UBE2C KIF20B KIAA0101 FOXM1 RACGAP1 GINS1 RRM2 MYBL2 ZWINT PLK1 CDCA8 HJURP KIF4A DTL NCAPH NCAPG CENPF NUSAP1 CCNB2 KIF23 CCDC109B CDK1 TOP2A

	LMNB1 NEK2 PRC1 BUB1 KIF14 CCNA2 CENPE POLQ HMMR TPX2 PBK CEP55 MKI67 CCNB1 KIF20A TMPO KIF2C NDC80 DLGAP5 ASPM
Elvidge Hypoxia by DMOG Up	DTNA VLDLR S100A4 SCNN1B MXI1 AHNAK2 DSC2 SPRY1 PLAUR PFKP SRD5A3 BNIP3L KLF6 NDRG1 EGFR EGLN1 CITED2 KLF7 FAM13A IGFBP3 ANG BNIP3 CAV1 KDM3A TMEM45A SFXN3 SAMD4A P4HA1 SOX9 PDK1 VEGFC FAM162A GYS1 ERO1L TXNIP SLCO4A1 PGK1 SLC2A1 ASPH JUN ALDOC PAM HK2 GJA1 TGFBI SRPX ENO2 ANGPTL4 LOX LOXL2 ITPR1 EGR1 EGLN3 CA9 STC1
Chang Cycling Genes	PWP1 BARD1 KDM5B MCM5 FAM72B SDC1 CASP3 HN1 DHFR ARL4A ANP32E CCNF ASF1B WDR76 SCML1 WSB1 GINS3 TACC3 TUBA4A CKAP2 MAD2L1 ATAD2 HELLS NCAPD2 UHRF1 MELK TUBB4B CENPA CDKN3 NEAT1 TRIP13 PCNA DEPDC1 AURKA MCM6 KIF22 KIFC1 H2AFX ESCO2 PTTG1 RAD51AP1 FAM83D DEPDC1B UBE2C KIF20B KIAA0101 IFIT1 FOXM1 CDCA7 RRM2 CDC25C PLK1 DIAPH3 CDCA8 HJURP NCAPH CENPF NUSAP1 CCNB2 KIF23 CDK1 TOP2A LMNB1 BUB1 SKA3 CCNA2 CKAP2L GAS2L3 HMMR TPX2 PBK NUF2 FAM111B DLGAP5 PRR11 ANLN
Chiang Liver Cancer Subclass Proliferation Up	PLBD1 OIP5 PM20D2 WSB1 MMP9 CDCA7L TUBA4A ETV4 SPHK1 MAD2L1 MEP1A CDC7 HELLS CDC20 AURKB FUNDC1 E2F8 NCEH1 CDKN3 TRIP13 SKA1 DEPDC1 ARHGAP18 AURKA TMEM51 FANCI HIST1H4C ORC6 SOX4 TTK LAMB1 PTTG1 BACE2 SHCBP1 RAD51AP1 MARCKSL1 MMP12 DEPDC1B UBE2C SLC39A10 FOXM1 RACGAP1 UGCG CDCA7 ZWINT PELI1 HJURP TRNP1 KIF4A DTL NCAPG CENPF SOX9 NUSAP1 CCNB2 KIF23 CCDC109B CDK1 TOP2A LMNB1 NEK2 PRC1 KIF14 CCNA2 CKAP2L CENPE TPX2 PBK CEP55 MKI67 NUF2 CCNB1 KIF20A HK2 KIF2C PAG1 NDC80 DLGAP5 PRR11 ANLN ASPM

REFERENCES

1 Haas, G. P., Delongchamps, N., Brawley, O. W., Wang, C. Y. & de la Roza, G. The worldwide epidemiology of prostate cancer: perspectives from autopsy studies. *The Canadian journal of urology* **15**, 3866-3871 (2008).

2 Society, A. C. *Cancer Facts & Figures 2018.*, <https://www.cancer.org/cancer/prostate-cancer/about/key-statistics.html#references> (2018).

3 Siegel, R. L., Miller, K. D. & Jemal, A. Cancer statistics, 2018. *CA: a cancer journal for clinicians* **68**, 7-30, doi:10.3322/caac.21442 (2018).

4 Yin, M., Bastacky, S., Chandran, U., Becich, M. J. & Dhir, R. Prevalence of incidental prostate cancer in the general population: a study of healthy organ donors. *The Journal of urology* **179**, 892-895; discussion 895, doi:10.1016/j.juro.2007.10.057 (2008).

5 Institute, N. C. *Cancer Statistics Factsheets S EER Prostate Cancer*, <http://seer.cancer.gov/statfacts/html/prost.html> (2018).

6 Parkin, D. M., Bray, F. I. & Devesa, S. S. Cancer burden in the year 2000. The global picture. *European journal of cancer* **37 Suppl 8**, S4-66 (2001).

7 DeSantis, C. E. *et al.* Cancer statistics for African Americans, 2016: Progress and opportunities in reducing racial disparities. *CA: a cancer journal for clinicians* **66**, 290-308, doi:10.3322/caac.21340 (2016).

8 Hsing, A. W., Sakoda, L. C. & Chua, S., Jr. Obesity, metabolic syndrome, and prostate cancer. *The American journal of clinical nutrition* **86**, s843-857 (2007).

9 Giri, V. N. *et al.* Association between agent orange and prostate cancer: A pilot case-control study. *Urology* **63**, 757-760, doi:10.1016/j.urology.2003.11.044 (2004).

10 Bhindi, B. *et al.* Dissecting the Association Between Metabolic Syndrome and Prostate Cancer Risk: Analysis of a Large Clinical Cohort. *Eur Urol* **67**, 64-70, doi:10.1016/j.eururo.2014.01.040 (2015).

11 Gathirua-Mwangi, W. G. & Zhang, J. Dietary factors and risk for advanced prostate cancer. *European journal of cancer prevention : the official journal of the European Cancer Prevention Organisation* **23**, 96-109, doi:10.1097/CEJ.0b013e3283647394 (2014).

12 Markozannes, G. *et al.* Diet, body size, physical activity and risk of prostate cancer: An umbrella review of the evidence. *European journal of cancer* **69**, 61-69, doi:10.1016/j.ejca.2016.09.026 (2016).

13 Wilson, K. M., Giovannucci, E. L. & Mucci, L. A. Lifestyle and dietary factors in the prevention of lethal prostate cancer. *Asian journal of andrology* **14**, 365-374, doi:10.1038/aja.2011.142 (2012).

14 Giovannucci, E., Liu, Y., Platz, E. A., Stampfer, M. J. & Willett, W. C. Risk factors for prostate cancer incidence and progression in the health professionals follow-up study. *Int J Cancer* **121**, 1571-1578, doi:10.1002/ijc.22788 (2007).

15 Crawford, E. D. Epidemiology of prostate cancer. *Urology* **62**, 3-12 (2003).

16 Chan, J. M., Stampfer, M. J. & Giovannucci, E. L. What causes prostate cancer? A brief summary of the epidemiology. *Seminars in cancer biology* **8**, 263-273 (1998).

17 Noone AM, H. N., Krapcho M, Miller D, Brest A, Yu M, Ruhl J, Tatalovich Z, Mariotto A, Lewis DR, Chen HS, Feuer EJ, Cronin KA (ed National Cancer Institute.) (2018).

18 Oh, B. *et al.* Oxidative stress in prostate cancer patients: A systematic review of case control studies. *Prostate international* **4**, 71-87, doi:10.1016/j.prnil.2016.05.002 (2016).

19 De Marzo, A. M. *et al.* Inflammation in prostate carcinogenesis. *Nature reviews. Cancer* **7**, 256-269, doi:10.1038/nrc2090 (2007).

20 Andriole, G. *et al.* Chemoprevention of prostate cancer in men at high risk: Rationale and design of the reduction by dutasteride of prostate cancer events (reduce) trial. *J Urology* **172**, 1314-1317, doi:10.1097/01.ju.0000139320.78673.2.a (2004).

21 Nickel, J. C. *et al.* The Relationship between Prostate Inflammation and Lower Urinary Tract Symptoms: Examination of Baseline Data from the REDUCE Trial. *Eur Urol* **54**, 1379-1384, doi:10.1016/j.eururo.2007.11.026 (2008).

22 Jacobs, E. J. *et al.* A large cohort study of aspirin and other nonsteroidal anti-inflammatory drugs and prostate cancer incidence. *Journal of the National Cancer Institute* **97**, 975-980, doi:10.1093/jnci/dji173 (2005).

23 Pruthi, R. S., Derksen, J. E. & Moore, D. A pilot study of use of the cyclooxygenase-2 inhibitor celecoxib in recurrent prostate cancer after definitive radiation therapy or radical prostatectomy. *BJU international* **93**, 275-278, doi:10.1111/j.1464-410X.2004.04601.x (2004).

24 Di Silverio, F., Sciarra, A. & Gentile, V. Etoricoxib and intermittent androgen deprivation therapy in patients with biochemical progression after radical prostatectomy. *Urology* **71**, 947-951, doi:10.1016/j.urology.2007.09.033 (2008).

25 Venkatesh Vaidyanathan, N. K., Anower Jabed , Radha Pallati, Chi Hsiu-Juei Kao, Alice Wang, Gareth Marlow, and Lynnette R. Ferguson. Prostate Cancer: Is It a Battle Lost to Age? *Geriatrics* **1** (2016).

26 Aunan, J. R., Cho, W. C. & Soreide, K. The Biology of Aging and Cancer: A Brief Overview of Shared and Divergent Molecular Hallmarks. *Aging and disease* **8**, 628-642, doi:10.14336/AD.2017.0103 (2017).

27 Thompson, I. *et al.* Association of African-American ethnic background with survival in men with metastatic prostate cancer. *Journal of the National Cancer Institute* **93**, 219-225 (2001).

28 Parker, P. M. *et al.* Prostate Cancer in Men Less Than the Age of 50: A Comparison of Race and Outcomes. *Urology* **78**, 110-115, doi:10.1016/j.urology.2010.12.046 (2011).

29 Hoffman, R. M. *et al.* Racial and ethnic differences in advanced-stage prostate cancer: the Prostate Cancer Outcomes Study. *Journal of the National Cancer Institute* **93**, 388-395 (2001).

30 Powell, I. J. *et al.* Should African-American men be tested for prostate carcinoma at an earlier age than white men? *Cancer* **85**, 472-477 (1999).

31 Cross, C. K. *et al.* Impact of race on prostate-specific antigen outcome after radical prostatectomy for clinically localized adenocarcinoma of the prostate. *Journal of Clinical Oncology* **20**, 2863-2868, doi:10.1200/Jco.2002.11.054 (2002).

32 Hamilton, R. J. *et al.* Race, biochemical disease recurrence, and prostate-specific antigen doubling time after radical prostatectomy: results from the SEARCH database. *Cancer* **110**, 2202-2209, doi:10.1002/cncr.23012 (2007).

33 Kumar, S., Singh, R., Malik, S., Manne, U. & Mishra, M. Prostate cancer health disparities: An immuno-biological perspective. *Cancer letters* **414**, 153-165, doi:10.1016/j.canlet.2017.11.011 (2018).

34 Bhardwaj, A. *et al.* Racial disparities in prostate cancer: a molecular perspective. *Frontiers in bioscience* **22**, 772-782 (2017).

35 Singh, S. K., Lillard, J. W., Jr. & Singh, R. Molecular basis for prostate cancer racial disparities. *Frontiers in bioscience* **22**, 428-450 (2017).

36 Goldgar, D. E., Easton, D. F., Cannon-Albright, L. A. & Skolnick, M. H. Systematic population-based assessment of cancer risk in first-degree relatives of cancer probands. *Journal of the National Cancer Institute* **86**, 1600-1608 (1994).

37 Schaid, D. J. The complex genetic epidemiology of prostate cancer. *Human molecular genetics* **13 Spec No 1**, R103-121, doi:10.1093/hmg/ddh072 (2004).

38 Powell, I. J. The precise role of ethnicity and family history on aggressive prostate cancer: a review analysis. *Archivos espanoles de urologia* **64**, 711-719 (2011).

39 Johns, L. E. & Houlston, R. S. A systematic review and meta-analysis of familial prostate cancer risk. *BJU international* **91**, 789-794 (2003).

40 Carter, B. S. *et al.* Hereditary prostate cancer: epidemiologic and clinical features. *The Journal of urology* **150**, 797-802 (1993).

41 Lichtenstein, P. *et al.* Environmental and heritable factors in the causation of cancer--analyses of cohorts of twins from Sweden, Denmark, and Finland. *The New England journal of medicine* **343**, 78-85, doi:10.1056/NEJM200007133430201 (2000).

42 Gronberg, H., Damber, L. & Damber, J. E. Studies of genetic factors in prostate cancer in a twin population. *The Journal of urology* **152**, 1484-1487; discussion 1487-1489 (1994).

43 Lee, J., Demissie, K., Lu, S. E. & Rhoads, G. G. Cancer incidence among Korean-American immigrants in the United States and native Koreans in South Korea. *Cancer control : journal of the Moffitt Cancer Center* **14**, 78-85, doi:10.1177/107327480701400111 (2007).

44 Ostrander, E. A., Markianos, K. & Stanford, J. L. Finding prostate cancer susceptibility genes. *Annual review of genomics and human genetics* **5**, 151-175, doi:10.1146/annurev.genom.5.061903.180044 (2004).

45 Lu, Y. *et al.* Most common 'sporadic' cancers have a significant germline genetic component. *Human molecular genetics* **23**, 6112-6118, doi:10.1093/hmg/ddu312 (2014).

46 Bratt, O., Kristoffersson, U., Olsson, H. & Lundgren, R. Clinical course of early onset prostate cancer with special reference to family history as a prognostic factor. *Eur Urol* **34**, 19-24, doi:Doi 10.1159/000019672 (1998).

47 Bratt, O., Damber, J. E., Emanuelsson, M. & Gronberg, H. Hereditary prostate cancer: clinical characteristics and survival. *The Journal of urology* **167**, 2423-2426 (2002).

48 Eeles, R. *et al.* The genetic epidemiology of prostate cancer and its clinical implications. *Nature reviews. Urology* **11**, 18-31, doi:10.1038/nrurol.2013.266 (2014).

49 Cui, J. *et al.* Segregation analyses of 1,476 population-based Australian families affected by prostate cancer. *American journal of human genetics* **68**, 1207-1218, doi:10.1086/320114 (2001).

50 Carter, B. S., Beaty, T. H., Steinberg, G. D., Childs, B. & Walsh, P. C. Mendelian inheritance of familial prostate cancer. *Proceedings of the National Academy of Sciences of the United States of America* **89**, 3367-3371 (1992).

51 Gronberg, H., Damber, L., Damber, J. E. & Iselius, L. Segregation analysis of prostate cancer in Sweden: support for dominant inheritance. *American journal of epidemiology* **146**, 552-557 (1997).

52 Schaid, D. J., McDonnell, S. K., Blute, M. L. & Thibodeau, S. N. Evidence for autosomal dominant inheritance of prostate cancer. *American journal of human genetics* **62**, 1425-1438, doi:10.1086/301862 (1998).

53 Valeri, A. *et al.* Segregation analysis of prostate cancer in France: evidence for autosomal dominant inheritance and residual brother-brother dependence. *Annals of human genetics* **67**, 125-137 (2003).

54 Verhage, B. A. *et al.* Autosomal dominant inheritance of prostate cancer: a confirmatory study. *Urology* **57**, 97-101 (2001).

55 Conlon, E. M. *et al.* Oligogenic segregation analysis of hereditary prostate cancer pedigrees: Evidence for multiple loci affecting age at onset. *Int J Cancer* **105**, 630-635, doi:10.1002/ijc.11128 (2003).

56 Gong, G. *et al.* Segregation analysis of prostate cancer in 1,719 white, African-American and Asian-American families in the United States and Canada. *Cancer causes & control : CCC* **13**, 471-482 (2002).

57 MacInnis, R. J. *et al.* Prostate Cancer Segregation Analyses Using 4390 Families from UK and Australian Population-Based Studies. *Genet Epidemiol* **34**, 42-50, doi:10.1002/gepi.20433 (2010).

58 Ewing, C. M. *et al.* Germline mutations in HOXB13 and prostate-cancer risk. *The New England journal of medicine* **366**, 141-149, doi:10.1056/NEJMoa1110000 (2012).

59 Tavtigian, S. V. *et al.* A candidate prostate cancer susceptibility gene at chromosome 17p. *Nature genetics* **27**, 172-180, doi:Doi 10.1038/84808 (2001).

60 Carpten, J. *et al.* Germline mutations in the ribonuclease L gene in families showing linkage with HPC1. *Nature genetics* **30**, 181-184, doi:10.1038/ng823 (2002).

61 Xu, J. *et al.* Evaluation of linkage and association of HPC2/ELAC2 in patients with familial or sporadic prostate cancer. *American journal of human genetics* **68**, 901-911 (2001).

62 Edwards, S. M. *et al.* Two percent of men with early-onset prostate cancer harbor germline mutations in the BRCA2 gene. *American journal of human genetics* **72**, 1-12 (2003).

63 Kote-Jarai, Z. *et al.* BRCA2 is a moderate penetrance gene contributing to young-onset prostate cancer: implications for genetic testing in prostate cancer patients. *British journal of cancer* **105**, 1230-1234, doi:10.1038/bjc.2011.383 (2011).

64 Leongamornlert, D. *et al.* Germline BRCA1 mutations increase prostate cancer risk. *British journal of cancer* **106**, 1697-1701, doi:10.1038/bjc.2012.146 (2012).

65 Hope, Q. *et al.* Macrophage scavenger receptor 1 999C>T (R293X) mutation and risk of prostate cancer. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology* **14**, 397-402, doi:10.1158/1055-9965.EPI-04-0202 (2005).

66 Cancel-Tassin, G. *et al.* PCAP is the major known prostate cancer predisposing locus in families from south and west Europe. *Eur J Hum Genet* **9**, 135-142, doi:DOI 10.1038/sj.ejhg.5200592 (2001).

67 Berthon, P. *et al.* Predisposing gene for early-onset prostate cancer, localized on chromosome 1q42.2-43. *American journal of human genetics* **62**, 1416-1424 (1998).

68 Gibbs, M. *et al.* Evidence for a rare prostate cancer-susceptibility locus at chromosome 1p36. *American journal of human genetics* **64**, 776-787, doi:Doi 10.1086/302287 (1999).

69 Colloca, G. & Venturino, A. The evolving role of familial history for prostate cancer. *Acta oncologica* **50**, 14-24, doi:10.3109/0284186X.2010.521191 (2011).

70 Benafif, S., Kote-Jarai, Z., Eeles, R. A. & Consortium, P. A Review of Prostate Cancer Genome-Wide Association Studies (GWAS). *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology* **27**, 845-857, doi:10.1158/1055-9965.EPI-16-1046 (2018).

71 Knudsen, B. S. & Vasioukhin, V. Mechanisms of prostate cancer initiation and progression. *Advances in cancer research* **109**, 1-50, doi:10.1016/B978-0-12-380890-5.00001-6 (2010).

72 Verze, P., Cai, T. & Lorenzetti, S. The role of the prostate in male fertility, health and disease. *Nature reviews. Urology* **13**, 379-386, doi:10.1038/nrurol.2016.89 (2016).

73 Shen, M. M. & Abate-Shen, C. Molecular genetics of prostate cancer: new prospects for old challenges. *Genes & development* **24**, 1967-2000, doi:10.1101/gad.1965810 (2010).

74 Timms, B. G. Prostate development: a historical perspective. *Differentiation; research in biological diversity* **76**, 565-577, doi:10.1111/j.1432-0436.2008.00278.x (2008).

75 De Marzo, A. M., Haffner, M. C., Lotan, T. L., Yegnasubramanian, S. & Nelson, W. G. Premalignancy in Prostate Cancer: Rethinking What we Know. *Cancer prevention research* **9**, 648-656, doi:10.1158/1940-6207.CAPR-15-0431 (2016).

76 McNeal, J. E. Normal histology of the prostate. *The American journal of surgical pathology* **12**, 619-633 (1988).

77 Wang, W., Bergh, A. & Damber, J. E. Morphological transition of proliferative inflammatory atrophy to high-grade intraepithelial neoplasia and cancer in human prostate. *The Prostate* **69**, 1378-1386, doi:10.1002/pros.20992 (2009).

78 De Marzo, A. M., Marchi, V. L., Epstein, J. I. & Nelson, W. G. Proliferative inflammatory atrophy of the prostate: implications for prostatic carcinogenesis. *The American journal of pathology* **155**, 1985-1992, doi:10.1016/S0002-9440(10)65517-4 (1999).

79 Putzi, M. J. & De Marzo, A. M. Morphologic transitions between proliferative inflammatory atrophy and high-grade prostatic intraepithelial neoplasia. *Urology* **56**, 828-832, doi:Doi 10.1016/S0090-4295(00)00776-7 (2000).

80 Abate-Shen, C. & Shen, M. M. Molecular genetics of prostate cancer. *Genes & development* **14**, 2410-2434, doi:Doi 10.1101/Gad.819500 (2000).

81 Bostwick, D. G., Liu, L., Brawer, M. K. & Qian, J. High-grade prostatic intraepithelial neoplasia. *Reviews in urology* **6**, 171-179 (2004).

82 Nagle, R. B., Brawer, M. K., Kittelson, J. & Clark, V. Phenotypic relationships of prostatic intraepithelial neoplasia to invasive prostatic carcinoma. *The American journal of pathology* **138**, 119-128 (1991).

83 Merrimen, J. L., Evans, A. J. & Srigley, J. R. Preneoplasia in the prostate gland with emphasis on high grade prostatic intraepithelial neoplasia. *Pathology* **45**, 251-263, doi:10.1097/PAT.0b013e32835f6134 (2013).

84 Bostwick, D. G. & Brawer, M. K. Prostatic intra-epithelial neoplasia and early invasion in prostate cancer. *Cancer* **59**, 788-794 (1987).

85 Bostwick, D. G., Pacelli, A. & Lopez-Beltran, A. Molecular biology of prostatic intraepithelial neoplasia. *The Prostate* **29**, 117-134, doi:10.1002/(SICI)1097-0045(199608)29:2<117::AID-PROS7>3.0.CO;2-C (1996).

86 Baca, S. C. *et al.* Punctuated evolution of prostate cancer genomes. *Cell* **153**, 666-677, doi:10.1016/j.cell.2013.03.021 (2013).

87 Boysen, G. *et al.* SPOP mutation leads to genomic instability in prostate cancer. *eLife* **4**, doi:10.7554/eLife.09207 (2015).

88 Barbieri, C. E. *et al.* Exome sequencing identifies recurrent SPOP, FOXA1 and MED12 mutations in prostate cancer. *Nature genetics* **44**, 685-689, doi:10.1038/ng.2279 (2012).

89 Cerveira, N. *et al.* TMPRSS2-ERG gene fusion causing ERG overexpression precedes chromosome copy number changes in prostate carcinomas and paired HGPIN lesions. *Neoplasia* **8**, 826-832, doi:10.1593/neo.06427 (2006).
90 Lee, W. H. *et al.* Cytidine methylation of regulatory sequences near the pi-class glutathione S-transferase gene accompanies human prostatic carcinogenesis. *Proceedings of the National Academy of Sciences of the United States of America* **91**, 11733-11737 (1994).
91 Haffner, M. C. *et al.* Molecular evidence that invasive adenocarcinoma can mimic prostatic intraepithelial neoplasia (PIN) and intraductal carcinoma through retrograde glandular colonization. *J Pathol* **238**, 31-41, doi:10.1002/path.4628 (2016).
92 Haffner, M. C. & Barbieri, C. E. Shifting Paradigms for High-grade Prostatic Intraepithelial Neoplasia. *Eur Urol* **69**, 831-833, doi:10.1016/j.eururo.2015.11.020 (2016).
93 Gregory P. Swanson, A. T. K., Courtney N. Shaver, Yolanda Munoz Maldonado, T. Phillip Reilly. High Grade Prostatic Intraepithelial Neoplasia and the Risk of Prostate Cancer. *Open Journal of Urology* **8**, 67-76 (2018).
94 Park, K. *et al.* TMPRSS2:ERG gene fusion predicts subsequent detection of prostate cancer in patients with high-grade prostatic intraepithelial neoplasia. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* **32**, 206-211, doi:10.1200/JCO.2013.49.8386 (2014).
95 Dotto, G. P. Multifocal epithelial tumors and field cancerization: stroma as a primary determinant. *Journal of Clinical Investigation* **124**, 1446-1453, doi:10.1172/JCI72589 (2014).
96 Tyekucheva, S. *et al.* Stromal and epithelial transcriptional map of initiation progression and metastatic potential of human prostate cancer. *Nature communications* **8**, 420, doi:10.1038/s41467-017-00460-4 (2017).
97 Baca, S. C. & Garraway, L. A. The genomic landscape of prostate cancer. *Frontiers in endocrinology* **3**, 69, doi:10.3389/fendo.2012.00069 (2012).
98 Spans, L. *et al.* The genomic landscape of prostate cancer. *International journal of molecular sciences* **14**, 10822-10851, doi:10.3390/ijms140610822 (2013).
99 Moorcraft, S. Y., Gonzalez, D. & Walker, B. A. Understanding next generation sequencing in oncology: A guide for oncologists. *Critical reviews in oncology/hematology* **96**, 463-474, doi:10.1016/j.critrevonc.2015.06.007 (2015).
100 Barbieri, C. E. & Rubin, M. A. Genomic rearrangements in prostate cancer. *Current opinion in urology* **25**, 71-76, doi:10.1097/MOU.0000000000000129 (2015).
101 Kan, Z. *et al.* Diverse somatic mutation patterns and pathway alterations in human cancers. *Nature* **466**, 869-873, doi:10.1038/nature09208 (2010).
102 Cancer Genome Atlas Research, N. The Molecular Taxonomy of Primary Prostate Cancer. *Cell* **163**, 1011-1025, doi:10.1016/j.cell.2015.10.025 (2015).
103 Berger, M. F. *et al.* The genomic complexity of primary human prostate cancer. *Nature* **470**, 214-220, doi:10.1038/nature09744 (2011).
104 Mitchell, T. & Neal, D. E. The genomic evolution of human prostate cancer. *British journal of cancer* **113**, 193-198, doi:10.1038/bjc.2015.234 (2015).
105 Forbes, S. A. *et al.* COSMIC: somatic cancer genetics at high-resolution. *Nucleic acids research* **45**, D777-D783, doi:10.1093/nar/gkw1121 (2017).
106 Weischenfeldt, J. *et al.* Integrative genomic analyses reveal an androgen-driven somatic alteration landscape in early-onset prostate cancer. *Cancer cell* **23**, 159-170, doi:10.1016/j.ccr.2013.01.002 (2013).

107 Lalonde, E. *et al.* Tumour genomic and microenvironmental heterogeneity for integrated prediction of 5-year biochemical recurrence of prostate cancer: a retrospective cohort study. *The Lancet. Oncology* **15**, 1521-1532, doi:10.1016/S1470-2045(14)71021-6 (2014).

108 Hieronymus, H. *et al.* Copy number alteration burden predicts prostate cancer relapse. *Proceedings of the National Academy of Sciences of the United States of America* **111**, 11139-11144, doi:10.1073/pnas.1411446111 (2014).

109 Liu, W. N. DNA alterations in the tumor genome and their associations with clinical outcome in prostate cancer. *Asian journal of andrology* **18**, 533-542, doi:10.4103/1008-682X.177120 (2016).

110 Knudson, A. G. Two genetic hits (more or less) to cancer. *Nature reviews. Cancer* **1**, 157-162, doi:10.1038/35101031 (2001).

111 Knudson, A. G., Jr., Meadows, A. T., Nichols, W. W. & Hill, R. Chromosomal deletion and retinoblastoma. *The New England journal of medicine* **295**, 1120-1123, doi:10.1056/NEJM197611112952007 (1976).

112 Morris, L. G. & Chan, T. A. Therapeutic targeting of tumor suppressor genes. *Cancer* **121**, 1357-1368, doi:10.1002/cncr.29140 (2015).

113 Berger, A. H., Knudson, A. G. & Pandolfi, P. P. A continuum model for tumour suppression. *Nature* **476**, 163-169, doi:10.1038/nature10275 (2011).

114 Dong, J. T. Chromosomal deletions and tumor suppressor genes in prostate cancer. *Cancer metastasis reviews* **20**, 173-193 (2001).

115 Shindo, T. *et al.* Kruppel-like zinc-finger transcription factor KLF5/BTEB2 is a target for angiotensin II signaling and an essential regulator of cardiovascular remodeling. *Nature medicine* **8**, 856-863, doi:10.1038/nm738 (2002).

116 Liu, W. *et al.* Homozygous deletions and recurrent amplifications implicate new genes involved in prostate cancer. *Neoplasia* **10**, 897-907 (2008).

117 Zhang, H. *et al.* SEECancer: a resource for somatic events in evolution of cancer genome. *Nucleic acids research* **46**, D1018-D1026, doi:10.1093/nar/gkx964 (2018).

118 Kaffenberger, S. D. & Barbieri, C. E. Molecular subtyping of prostate cancer. *Current opinion in urology* **26**, 213-218, doi:10.1097/MOU.0000000000000285 (2016).

119 Nakayama, M. *et al.* Hypermethylation of the human glutathione S-transferase-pi gene (GSTP1) CpG island is present in a subset of proliferative inflammatory atrophy lesions but not in normal or hyperplastic epithelium of the prostate: a detailed study using laser-capture microdissection. *The American journal of pathology* **163**, 923-933 (2003).

120 Yegnasubramanian, S. *et al.* Hypermethylation of CpG islands in primary and metastatic human prostate cancer. *Cancer research* **64**, 1975-1986 (2004).

121 Yegnasubramanian, S. Prostate cancer epigenetics and its clinical implications. *Asian journal of andrology* **18**, 549-558, doi:10.4103/1008-682X.179859 (2016).

122 Yegnasubramanian, S. *et al.* DNA Hypomethylation Arises Later in Prostate Cancer Progression than CpG Island Hypermethylation and Contributes to Metastatic Tumor Heterogeneity. *Cancer research* **68**, 8954-8967, doi:10.1158/0008-5471.CAN-07-6088 (2008).

123 Malinen, M., Niskanen, E. A., Kaikkonen, M. U. & Palvimo, J. J. Crosstalk between androgen and pro-inflammatory signaling remodels androgen receptor and NF-kappa B cistrome to reprogram the prostate cancer cell transcriptome. *Nucleic acids research* **45**, 619-630, doi:10.1093/nar/gkw855 (2017).

124 Sfanos, K. S., Hempel, H. A. & De Marzo, A. M. The role of inflammation in prostate cancer. *Advances in experimental medicine and biology* **816**, 153-181, doi:10.1007/978-3-0348-0837-8_7 (2014).
125 Thapa, D. & Ghosh, R. Chronic inflammatory mediators enhance prostate cancer development and progression. *Biochemical pharmacology* **94**, 53-62, doi:10.1016/j.bcp.2014.12.023 (2015).
126 Sfanos, K. S. & De Marzo, A. M. Prostate cancer and inflammation: the evidence. *Histopathology* **60**, 199-215, doi:10.1111/j.1365-2559.2011.04033.x (2012).
127 Dasgupta, K. *et al.* Association between nonsteroidal anti-inflammatory drugs and prostate cancer occurrence. *Cancer J* **12**, 130-135 (2006).
128 Nelson, J. E. & Harris, R. E. Inverse association of prostate cancer and non-steroidal anti-inflammatory drugs (NSAIDs): results of a case-control study. *Oncology reports* **7**, 169-170 (2000).
129 Roberts, R. O. *et al.* A population-based study of daily nonsteroidal anti-inflammatory drug use and prostate cancer. *Mayo Clin Proc* **77**, 219-225, doi:Doi 10.4065/77.3.219 (2002).
130 Mahmud, S. M., Franco, E. L. & Aprikian, A. G. Use of nonsteroidal anti-inflammatory drugs and prostate cancer risk: a meta-analysis. *Int J Cancer* **127**, 1680-1691, doi:10.1002/ijc.25186 (2010).
131 Green, S. M., Mostaghel, E. A. & Nelson, P. S. Androgen action and metabolism in prostate cancer. *Molecular and cellular endocrinology* **360**, 3-13, doi:10.1016/j.mce.2011.09.046 (2012).
132 Wang, F. & Koul, H. K. Androgen receptor (AR) cistrome in prostate differentiation and cancer progression. *American journal of clinical and experimental urology* **5**, 18-24 (2017).
133 Schuurmans, A. L. G., Bolt, J. & Mulder, E. Androgens Stimulate Both Growth-Rate and Epidermal Growth-Factor Receptor Activity of the Human-Prostate Tumor-Cell Lncap. *The Prostate* **12**, 55-63, doi:DOI 10.1002/pros.2990120108 (1988).
134 Silva, I. S., Morsch, D. M., Urnauer, L. & Spritzer, P. M. Androgen-induced cell growth and c-myc expression in human non-transformed epithelial prostatic cells in primary culture. *Endocrine research* **27**, 153-169 (2001).
135 Schroder, F., Crawford, E. D., Axcrona, K., Payne, H. & Keane, T. E. Androgen deprivation therapy: past, present and future. *BJU international* **109 Suppl 6**, 1-12, doi:10.1111/j.1464-410X.2012.11215.x (2012).
136 Chandrasekar, T., Yang, J. C., Gao, A. C. & Evans, C. P. Mechanisms of resistance in castration-resistant prostate cancer (CRPC). *Translational andrology and urology* **4**, 365-380, doi:10.3978/j.issn.2223-4683.2015.05.02 (2015).
137 Ward, P. S. & Thompson, C. B. Metabolic reprogramming: a cancer hallmark even warburg did not anticipate. *Cancer cell* **21**, 297-308, doi:10.1016/j.ccr.2012.02.014 (2012).
138 Hanahan, D. & Weinberg, R. A. Hallmarks of cancer: the next generation. *Cell* **144**, 646-674, doi:10.1016/j.cell.2011.02.013 (2011).
139 Pavlova, N. N. & Thompson, C. B. The Emerging Hallmarks of Cancer Metabolism. *Cell metabolism* **23**, 27-47, doi:10.1016/j.cmet.2015.12.006 (2016).
140 Martinez-Outschoorn, U. E., Peiris-Pages, M., Pestell, R. G., Sotgia, F. & Lisanti, M. P. Cancer metabolism: a therapeutic perspective. *Nature reviews. Clinical oncology* **14**, 113, doi:10.1038/nrclinonc.2017.1 (2017).
141 Koppenol, W. H., Bounds, P. L. & Dang, C. V. Otto Warburg's contributions to current concepts of cancer metabolism. *Nature reviews. Cancer* **11**, 325-337, doi:10.1038/nrc3038 (2011).

142 Lunt, S. Y. & Vander Heiden, M. G. Aerobic glycolysis: meeting the metabolic requirements of cell proliferation. *Annual review of cell and developmental biology* **27**, 441-464, doi:10.1146/annurev-cellbio-092910-154237 (2011).
143 Moreno-Sanchez, R., Rodriguez-Enriquez, S., Marin-Hernandez, A. & Saavedra, E. Energy metabolism in tumor cells. *Febs J* **274**, 1393-1418, doi:10.1111/j.1742-4658.2007.05686.x (2007).
144 Warburg, O. On the origin of cancer cells. *Science* **123**, 309-314 (1956).
145 Fantin, V. R., St-Pierre, J. & Leder, P. Attenuation of LDH-A expression uncovers a link between glycolysis, mitochondrial physiology, and tumor maintenance. *Cancer cell* **9**, 425-434, doi:10.1016/j.ccr.2006.04.023 (2006).
146 Zu, X. L. & Guppy, M. Cancer metabolism: facts, fantasy, and fiction. *Biochemical and biophysical research communications* **313**, 459-465 (2004).
147 Israelsen, W. J. *et al.* PKM2 isoform-specific deletion reveals a differential requirement for pyruvate kinase in tumor cells. *Cell* **155**, 397-409, doi:10.1016/j.cell.2013.09.025 (2013).
148 Joshi, S. *et al.* The Genomic Landscape of Renal Oncocytoma Identifies a Metabolic Barrier to Tumorigenesis. *Cell reports* **13**, 1895-1908, doi:10.1016/j.celrep.2015.10.059 (2015).
149 Weinberg, F. *et al.* Mitochondrial metabolism and ROS generation are essential for Kras-mediated tumorigenicity. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 8788-8793, doi:10.1073/pnas.1003428107 (2010).
150 Martinez-Reyes, I. *et al.* TCA Cycle and Mitochondrial Membrane Potential Are Necessary for Diverse Biological Functions. *Mol Cell* **61**, 199-209, doi:10.1016/j.molcel.2015.12.002 (2016).
151 DeBerardinis, R. J. & Chandel, N. S. Fundamentals of cancer metabolism. *Science advances* **2**, e1600200, doi:10.1126/sciadv.1600200 (2016).
152 Ahn, C. S. & Metallo, C. M. Mitochondria as biosynthetic factories for cancer proliferation. *Cancer Metab* **3**, doi:Artn 1

10.1186/S40170-015-0128-2 (2015).
153 Desideri, E., Vegliante, R. & Ciriolo, M. R. Mitochondrial dysfunctions in cancer: genetic defects and oncogenic signaling impinging on TCA cycle activity. *Cancer letters* **356**, 217-223, doi:10.1016/j.canlet.2014.02.023 (2015).
154 Locasale, J. W. Serine, glycine and one-carbon units: cancer metabolism in full circle. *Nature Reviews Cancer* **13**, 572-583, doi:10.1038/nrc3557 (2013).
155 Hay, N. Reprogramming glucose metabolism in cancer: can it be exploited for cancer therapy? *Nature reviews. Cancer* **16**, 635-649, doi:10.1038/nrc.2016.77 (2016).
156 Hosios, A. M. & Vander Heiden, M. G. The redox requirements of proliferating mammalian cells. *The Journal of biological chemistry*, doi:10.1074/jbc.TM117.000239 (2018).
157 Zachara, N. E. & Hart, G. W. O-GlcNAc a sensor of cellular state: the role of nucleocytoplasmic glycosylation in modulating cellular function in response to nutrition and stress. *Biochimica et biophysica acta* **1673**, 13-28, doi:10.1016/j.bbagen.2004.03.016 (2004).
158 Hirschey, M. D. *et al.* Dysregulated metabolism contributes to oncogenesis. *Seminars in cancer biology* **35 Suppl**, S129-S150, doi:10.1016/j.semcancer.2015.10.002 (2015).
159 Choi, Y. K. & Park, K. G. Targeting Glutamine Metabolism for Cancer Treatment. *Biomolecules & therapeutics* **26**, 19-28, doi:10.4062/biomolther.2017.178 (2018).

160 Murai, S. *et al.* Inhibition of malic enzyme 1 disrupts cellular metabolism and leads to vulnerability in cancer cells in glucose-restricted conditions. *Oncogenesis* **6**, e329, doi:10.1038/oncsis.2017.34 (2017).
161 DeBerardinis, R. J. *et al.* Beyond aerobic glycolysis: Transformed cells can engage in glutamine metabolism that exceeds the requirement for protein and nucleotide synthesis. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 19345-19350, doi:10.1073/pnas.0709747104 (2007).
162 Beloribi-Djefaflia, S., Vasseur, S. & Guillaumond, F. Lipid metabolic reprogramming in cancer cells. *Oncogenesis* **5**, e189, doi:10.1038/oncsis.2015.49 (2016).
163 Santos, C. R. & Schulze, A. Lipid metabolism in cancer. *Febs J* **279**, 2610-2623, doi:10.1111/j.1742-4658.2012.08644.x (2012).
164 Berg JM, T. J., Stryer L. in *Biochemistry* Vol. 5 (W H Freeman, New York, 2002).
165 Baenke, F., Peck, B., Miess, H. & Schulze, A. Hooked on fat: the role of lipid synthesis in cancer metabolism and tumour development. *Disease models & mechanisms* **6**, 1353-1363, doi:10.1242/dmm.011338 (2013).
166 Zadra, G., Photopoulos, C. & Loda, M. The fat side of prostate cancer. *Biochimica et biophysica acta* **1831**, 1518-1532, doi:10.1016/j.bbalip.2013.03.010 (2013).
167 Head, B. P., Patel, H. H. & Insel, P. A. Interaction of membrane/lipid rafts with the cytoskeleton: impact on signaling and function: membrane/lipid rafts, mediators of cytoskeletal arrangement and cell signaling. *Biochimica et biophysica acta* **1838**, 532-545, doi:10.1016/j.bbamem.2013.07.018 (2014).
168 Berg JM, T. J., Stryer L. . in *Biochemistry. 5th edition. Section 26.4* (W H Freeman, 2002).
169 Konstantinopoulos, P. A., Karamouzis, M. V. & Papavassiliou, A. G. Post-translational modifications and regulation of the RAS superfamily of GTPases as anticancer targets. *Nat Rev Drug Discov* **6**, 540-555, doi:10.1038/nrd2221 (2007).
170 Quetglas, J. I. *et al.* Small rho GTPases and cholesterol biosynthetic pathway intermediates in African swine fever virus infection. *Journal of virology* **86**, 1758-1767, doi:10.1128/JVI.05666-11 (2012).
171 Udenwobele, D. I. *et al.* Myristoylation: An Important Protein Modification in the Immune Response. *Frontiers in immunology* **8**, 751, doi:10.3389/fimmu.2017.00751 (2017).
172 Yang, C. *et al.* Glutamine oxidation maintains the TCA cycle and cell survival during impaired mitochondrial pyruvate transport. *Mol Cell* **56**, 414-424, doi:10.1016/j.molcel.2014.09.025 (2014).
173 Tricarico, P. M., Crovella, S. & Celsi, F. Mevalonate Pathway Blockade, Mitochondrial Dysfunction and Autophagy: A Possible Link. *International journal of molecular sciences* **16**, 16067-16084, doi:10.3390/ijms160716067 (2015).
174 Kunau, W. H., Dommes, V. & Schulz, H. beta-oxidation of fatty acids in mitochondria, peroxisomes, and bacteria: a century of continued progress. *Progress in lipid research* **34**, 267-342 (1995).
175 Carracedo, A., Cantley, L. C. & Pandolfi, P. P. Cancer metabolism: fatty acid oxidation in the limelight. *Nature reviews. Cancer* **13**, 227-232, doi:10.1038/nrc3483 (2013).
176 Schrader, M., Costello, J., Godinho, L. F. & Islinger, M. Peroxisome-mitochondria interplay and disease. *Journal of inherited metabolic disease* **38**, 681-702, doi:10.1007/s10545-015-9819-7 (2015).

177 Qu, Q., Zeng, F., Liu, X., Wang, Q. J. & Deng, F. Fatty acid oxidation and carnitine palmitoyltransferase I: emerging therapeutic targets in cancer. *Cell death & disease* **7**, e2226, doi:10.1038/cddis.2016.132 (2016).
178 Bonnefont, J. P. *et al.* Carnitine palmitoyltransferases 1 and 2: biochemical, molecular and medical aspects. *Molecular aspects of medicine* **25**, 495-520, doi:10.1016/j.mam.2004.06.004 (2004).
179 Berg JM, T. J., Stryer L. in *Biochemistry* (W H Freeman, 2002.).
180 Stine, Z. E., Walton, Z. E., Altman, B. J., Hsieh, A. L. & Dang, C. V. MYC, Metabolism, and Cancer. *Cancer Discov* **5**, 1024-1039, doi:10.1158/2159-8290.CD-15-0507 (2015).
181 Buzzai, M. *et al.* The glucose dependence of Akt-transformed cells can be reversed by pharmacologic activation of fatty acid beta-oxidation. *Oncogene* **24**, 4165-4173, doi:10.1038/sj.onc.1208622 (2005).
182 Elstrom, R. L. *et al.* Akt stimulates aerobic glycolysis in cancer cells. *Cancer research* **64**, 3892-3899, doi:10.1158/0008-5472.CAN-03-2904 (2004).
183 Porstmann, T. *et al.* SREBP activity is regulated by mTORC1 and contributes to Akt-dependent cell growth. *Cell metabolism* **8**, 224-236, doi:10.1016/j.cmet.2008.07.007 (2008).
184 Chen, C. H., Pore, N., Behrooz, A., Ismail-Beigi, F. & Maity, A. Regulation of glut1 mRNA by hypoxia-inducible factor-1 - Interaction between H-ras and hypoxia. *Journal of Biological Chemistry* **276**, 9519-9525, doi:DOI 10.1074/jbc.M010144200 (2001).
185 Mathupala, S. P., Rempel, A. & Pedersen, P. L. Glucose catabolism in cancer cells: identification and characterization of a marked activation response of the type II hexokinase gene to hypoxic conditions. *The Journal of biological chemistry* **276**, 43407-43412, doi:10.1074/jbc.M108181200 (2001).
186 King, A., Selak, M. A. & Gottlieb, E. Succinate dehydrogenase and fumarate hydratase: linking mitochondrial dysfunction and cancer. *Oncogene* **25**, 4675-4682, doi:10.1038/sj.onc.1209594 (2006).
187 DeBerardinis, R. J. Serine Metabolism: Some Tumors Take the Road Less Traveled. *Cell metabolism* **14**, 285-286, doi:10.1016/j.cmet.2011.08.004 (2011).
188 Luengo, A., Gui, D. Y. & Vander Heiden, M. G. Targeting Metabolism for Cancer Therapy. *Cell chemical biology* **24**, 1161-1180, doi:10.1016/j.chembiol.2017.08.028 (2017).
189 Chen, S. H. Asparaginase Therapy in Pediatric Acute Lymphoblastic Leukemia: A Focus on the Mode of Drug Resistance. *Pediatrics and neonatology* **56**, 287-293, doi:10.1016/j.pedneo.2014.10.006 (2015).
190 Ruggieri, V. *et al.* Dichloroacetate, a selective mitochondria-targeting drug for oral squamous cell carcinoma: a metabolic perspective of treatment. *Oncotarget* **6**, 1217-1230, doi:10.18632/oncotarget.2721 (2015).
191 Nilsson, R. *et al.* Metabolic enzyme expression highlights a key role for MTHFD2 and the mitochondrial folate pathway in cancer. *Nature communications* **5**, 3128, doi:10.1038/ncomms4128 (2014).
192 Koufaris, C. *et al.* Suppression of MTHFD2 in MCF-7 Breast Cancer Cells Increases Glycolysis, Dependency on Exogenous Glycine, and Sensitivity to Folate Depletion. *Journal of proteome research* **15**, 2618-2625, doi:10.1021/acs.jproteome.6b00188 (2016).
193 Selcuklu, S. D. *et al.* MicroRNA-9 inhibition of cell proliferation and identification of novel miR-9 targets by transcriptome profiling in breast cancer cells. *The Journal of biological chemistry* **287**, 29516-29528, doi:10.1074/jbc.M111.335943 (2012).

194 Ye, J. *et al.* Serine catabolism regulates mitochondrial redox control during hypoxia. *Cancer Discov* **4**, 1406-1417, doi:10.1158/2159-8290.CD-14-0250 (2014).
195 Linster, C. L. & Van Schaftingen, E. Vitamin C. Biosynthesis, recycling and degradation in mammals. *Febs J* **274**, 1-22, doi:10.1111/j.1742-4658.2006.05607.x (2007).
196 Yun, J. *et al.* Vitamin C selectively kills KRAS and BRAF mutant colorectal cancer cells by targeting GAPDH. *Science* **350**, 1391-1396, doi:10.1126/science.aaa5004 (2015).
197 Seoane, J. & De Mattos-Arruda, L. The challenge of intratumour heterogeneity in precision medicine. *Journal of internal medicine* **276**, 41-51, doi:10.1111/joim.12240 (2014).
198 Hensley, C. T. *et al.* Metabolic Heterogeneity in Human Lung Tumors. *Cell* **164**, 681-694, doi:10.1016/j.cell.2015.12.034 (2016).
199 Costello, L. C. & Franklin, R. B. The clinical relevance of the metabolism of prostate cancer; zinc and tumor suppression: connecting the dots. *Molecular cancer* **5**, 17, doi:10.1186/1476-4598-5-17 (2006).
200 Cutruzzola, F. *et al.* Glucose Metabolism in the Progression of Prostate Cancer. *Frontiers in physiology* **8**, 97, doi:10.3389/fphys.2017.00097 (2017).
201 Costello, L. C. *et al.* Human prostate cancer ZIP1/zinc/citrate genetic/metabolic relationship in the TRAMP prostate cancer animal model. *Cancer biology & therapy* **12**, 1078-1084, doi:10.4161/cbt.12.12.18367 (2011).
202 Franklin, R. B. *et al.* Human ZIP1 is a major zinc uptake transporter for the accumulation of zinc in prostate cells. *J Inorg Biochem* **96**, 435-442, doi:10.1016/S0162-0134(03)00249-6 (2003).
203 Huang, L., Kirschke, C. P. & Zhang, Y. Decreased intracellular zinc in human tumorigenic prostate epithelial cells: a possible role in prostate cancer progression. *Cancer cell international* **6**, 10, doi:10.1186/1475-2867-6-10 (2006).
204 Costello, L. C. & Franklin, R. B. Novel role of zinc in the regulation of prostate citrate metabolism and its implications in prostate cancer. *The Prostate* **35**, 285-296 (1998).
205 Tsui, K. H., Chang, P. L. & Juang, H. H. Zinc blocks gene expression of mitochondrial aconitase in human prostatic carcinoma cells. *Int J Cancer* **118**, 609-615, doi:10.1002/ijc.21411 (2006).
206 Fong, J. C., Lin, C. H., Wei, Y. H., Ho, L. T. & Hong, C. Y. Calcium buffering capacity of human seminal plasma: the role of EGTA in stimulating sperm motility. *The Chinese journal of physiology* **29**, 7-12 (1986).
207 Magnus, O., Abyholm, T., Kofstad, J. & Purvis, K. Ionized calcium in human male and female reproductive fluids: relationships to sperm motility. *Human reproduction* **5**, 94-98 (1990).
208 Arver, S. Zinc and zinc ligands in human seminal plasma. III. The principal low molecular weight zinc ligand in prostatic secretion and seminal plasma. *Acta physiologica Scandinavica* **116**, 67-73, doi:10.1111/j.1748-1716.1982.tb10600.x (1982).
209 Liu, Y. Fatty acid oxidation is a dominant bioenergetic pathway in prostate cancer. *Prostate cancer and prostatic diseases* **9**, 230-234, doi:10.1038/sj.pcan.4500879 (2006).
210 Chandler, J. D., Williams, E. D., Slavin, J. L., Best, J. D. & Rogers, S. Expression and localization of GLUT1 and GLUT12 in prostate carcinoma. *Cancer* **97**, 2035-2042, doi:10.1002/cncr.11293 (2003).
211 Oyama, N. *et al.* The increased accumulation of [18F]fluorodeoxyglucose in untreated prostate cancer. *Japanese journal of clinical oncology* **29**, 623-629 (1999).
212 Pertega-Gomes, N. *et al.* A glycolytic phenotype is associated with prostate cancer progression and aggressiveness: a role for monocarboxylate transporters as metabolic targets for therapy. *J Pathol* **236**, 517-530, doi:10.1002/path.4547 (2015).

213 Liu, Y., Zuckier, L. S. & Ghesani, N. V. Dominant uptake of fatty acid over glucose by prostate cells: a potential new diagnostic and therapeutic approach. *Anticancer research* **30**, 369-374 (2010).
214 Dueregger, A. *et al.* Differential Utilization of Dietary Fatty Acids in Benign and Malignant Cells of the Prostate. *PloS one* **10**, e0135704, doi:10.1371/journal.pone.0135704 (2015).
215 Krycer, J. R. & Brown, A. J. Cholesterol accumulation in prostate cancer: a classic observation from a modern perspective. *Biochimica et biophysica acta* **1835**, 219-229, doi:10.1016/j.bbcan.2013.01.002 (2013).
216 Franklin, R. B., Zou, J., Yu, Z. & Costello, L. C. EAAC1 is expressed in rat and human prostate epithelial cells; functions as a high-affinity L-aspartate transporter; and is regulated by prolactin and testosterone. *BMC biochemistry* **7**, 10, doi:10.1186/1471-2091-7-10 (2006).
217 Kolenko, V., Teper, E., Kutikov, A. & Uzzo, R. Zinc and zinc transporters in prostate carcinogenesis. *Nature reviews. Urology* **10**, 219-226, doi:10.1038/nrurol.2013.43 (2013).
218 Makhov, P. B. *et al.* Reversal of epigenetic silencing of AP-2alpha results in increased zinc uptake in DU-145 and LNCaP prostate cancer cells. *Carcinogenesis* **32**, 1773-1781, doi:10.1093/carcin/bgr212 (2011).
219 Uzzo, R. G. *et al.* Zinc inhibits nuclear factor-kappa B activation and sensitizes prostate cancer cells to cytotoxic agents. *Clin Cancer Res* **8**, 3579-3583 (2002).
220 Feng, P., Li, T. L., Guan, Z. X., Franklin, R. B. & Costello, L. C. Direct effect of zinc on mitochondrial apoptogenesis in prostate cells. *The Prostate* **52**, 311-318, doi:10.1002/pros.10128 (2002).
221 Uzzo, R. G. *et al.* Diverse effects of zinc on NF-kappaB and AP-1 transcription factors: implications for prostate cancer progression. *Carcinogenesis* **27**, 1980-1990, doi:10.1093/carcin/bgl034 (2006).
222 Singh, K. K., Desouki, M. M., Franklin, R. B. & Costello, L. C. Mitochondrial aconitase and citrate metabolism in malignant and nonmalignant human prostate tissues. *Molecular cancer* **5**, 14, doi:10.1186/1476-4598-5-14 (2006).
223 Costello, L. C. & Franklin, R. B. The intermediary metabolism of the prostate: a key to understanding the pathogenesis and progression of prostate malignancy. *Oncology* **59**, 269-282, doi:10.1159/000012183 (2000).
224 Cooper, J. F. & Farid, I. The Role of Citric Acid in the Physiology of the Prostate. 3. Lactate/Citrate Ratios in Benign and Malignant Prostatic Homogenates as an Index of Prostatic Malignancy. *The Journal of urology* **92**, 533-536 (1964).
225 Costello, L. C., Liu, Y., Franklin, R. B. & Kennedy, M. C. Zinc inhibition of mitochondrial aconitase and its importance in citrate metabolism of prostate epithelial cells. *The Journal of biological chemistry* **272**, 28875-28881 (1997).
226 Fiaschi, T. *et al.* Reciprocal Metabolic Reprogramming through Lactate Shuttle Coordinately Influences Tumor-Stroma Interplay. *Cancer research* **72**, 5130-5140, doi:10.1158/0008-5472.CAN-12-1949 (2012).
227 Valencia, T. *et al.* Metabolic Reprogramming of Stromal Fibroblasts through p62-mTORC1 Signaling Promotes Inflammation and Tumorigenesis. *Cancer cell* **26**, 121-135, doi:10.1016/j.ccr.2014.05.004 (2014).
228 Giannoni, E. *et al.* Targeting stromal-induced pyruvate kinase M2 nuclear translocation impairs oxphos and prostate cancer metastatic spread. *Oncotarget* **6**, 24061-24074, doi:10.18632/oncotarget.4448 (2015).

229 Zacharias, N. M. *et al.* Metabolic Differences in Glutamine Utilization Lead to Metabolic Vulnerabilities in Prostate Cancer. *Sci Rep* **7**, 16159, doi:10.1038/s41598-017-16327-z (2017).
230 Cluntun, A. A., Lukey, M. J., Cerione, R. A. & Locasale, J. W. Glutamine Metabolism in Cancer: Understanding the Heterogeneity. *Trends in cancer* **3**, 169-180, doi:10.1016/j.trecan.2017.01.005 (2017).
231 Pan, T. *et al.* Elevated expression of glutaminase confers glucose utilization via glutaminolysis in prostate cancer. *Biochemical and biophysical research communications* **456**, 452-458, doi:10.1016/j.bbrc.2014.11.105 (2015).
232 Gao, P. *et al.* c-Myc suppression of miR-23a/b enhances mitochondrial glutaminase expression and glutamine metabolism. *Nature* **458**, 762-765, doi:10.1038/nature07823 (2009).
233 Canape, C. *et al.* Probing treatment response of glutaminolytic prostate cancer cells to natural drugs with hyperpolarized [5-C-13]glutamine. *Magn Reson Med* **73**, 2296-2305, doi:10.1002/mrm.25360 (2015).
234 White, M. A. *et al.* Glutamine Transporters Are Targets of Multiple Oncogenic Signaling Pathways in Prostate Cancer. *Mol Cancer Res* **15**, 1017-1028, doi:10.1158/1541-7786.MCR-16-0480 (2017).
235 Wang, Q. *et al.* Targeting ASCT2-mediated glutamine uptake blocks prostate cancer growth and tumour development. *J Pathol* **236**, 278-289, doi:10.1002/path.4518 (2015).
236 Shafi, A. A. *et al.* Differential regulation of metabolic pathways by androgen receptor (AR) and its constitutively active splice variant, AR-V7, in prostate cancer cells. *Oncotarget* **6**, 31997-32012, doi:10.18632/oncotarget.5585 (2015).
237 Zhao, H. *et al.* Tumor microenvironment derived exosomes pleiotropically modulate cancer cell metabolism. *eLife* **5**, e10250, doi:10.7554/eLife.10250 (2016).
238 Al Kadhi, O. *et al.* Increased transcriptional and metabolic capacity for lipid metabolism in the peripheral zone of the prostate may underpin its increased susceptibility to cancer. *Oncotarget* **8**, 84902-84916, doi:10.18632/oncotarget.17926 (2017).
239 Rossi, S. *et al.* Fatty acid synthase expression defines distinct molecular signatures in prostate cancer. *Mol Cancer Res* **1**, 707-715 (2003).
240 Swinnen, J. V. *et al.* Overexpression of fatty acid synthase is an early and common event in the development of prostate cancer. *Int J Cancer* **98**, 19-22, doi:10.1002/ijc.10127 (2002).
241 Shurbaji, M. S., Kuhajda, F. P., Pasternack, G. R. & Thurmond, T. S. Expression of Oncogenic Antigen-519 (Oa-519) in Prostate-Cancer Is a Potential Prognostic Indicator. *Am J Clin Pathol* **97**, 686-691 (1992).
242 Shurbaji, M. S., Kalbfleisch, J. H. & Thurmond, T. S. Immunohistochemical detection of a fatty acid synthase (OA-519) as a predictor of progression of prostate cancer. *Hum Pathol* **27**, 917-921, doi:Doi 10.1016/S0046-8177(96)90218-X (1996).
243 Epstein, J. I., Carmichael, M. & Partin, A. W. OA-519 (fatty acid synthase) as an independent predictor of pathologic state in adenocarcinoma of the prostate. *Urology* **45**, 81-86 (1995).
244 Madigan, A. A. *et al.* Novel nuclear localization of fatty acid synthase correlates with prostate cancer aggressiveness. *The American journal of pathology* **184**, 2156-2162, doi:10.1016/j.ajpath.2014.04.012 (2014).
245 Migita, T. *et al.* Fatty acid synthase: a metabolic enzyme and candidate oncogene in prostate cancer. *Journal of the National Cancer Institute* **101**, 519-532, doi:10.1093/jnci/djp030 (2009).

246 Fiorentino, M. *et al.* Overexpression of fatty acid synthase is associated with palmitoylation of Wnt1 and cytoplasmic stabilization of beta-catenin in prostate cancer. *Lab Invest* **88**, 1340-1348, doi:10.1038/labinvest.2008.97 (2008).

247 Little, J. L., Wheeler, F. B., Fels, D. R., Koumenis, C. & Kridel, S. J. Inhibition of fatty acid synthase induces endoplasmic reticulum stress in tumor cells. *Cancer research* **67**, 1262-1269, doi:10.1158/0008-5472.CAN-06-1794 (2007).

248 Rysman, E. *et al.* De novo lipogenesis protects cancer cells from free radicals and chemotherapeutics by promoting membrane lipid saturation. *Cancer research* **70**, 8117-8126, doi:10.1158/0008-5472.CAN-09-3871 (2010).

249 Staubach, S. & Hanisch, F. G. Lipid rafts: signaling and sorting platforms of cells and their roles in cancer. *Expert review of proteomics* **8**, 263-277, doi:10.1586/epr.11.2 (2011).

250 De Schrijver, E., Brusselmans, K., Heyns, W., Verhoeven, G. & Swinnen, J. V. RNA interference-mediated silencing of the fatty acid synthase gene attenuates growth and induces morphological changes and apoptosis of LNCaP prostate cancer cells. *Cancer research* **63**, 3799-3804 (2003).

251 Rae, C., Haberkorn, U., Babich, J. W. & Mairs, R. J. Inhibition of Fatty Acid Synthase Sensitizes Prostate Cancer Cells to Radiotherapy. *Radiation research* **184**, 482-493, doi:10.1667/RR14173.1 (2015).

252 Alli, P. M., Pinn, M. L., Jaffee, E. M., McFadden, J. M. & Kuhajda, F. P. Fatty acid synthase inhibitors are chemopreventive for mammary cancer in neu-N transgenic mice. *Oncogene* **24**, 39-46, doi:10.1038/sj.onc.1208174 (2005).

253 Zhao, W. *et al.* Fatty acid synthase: a novel target for antiglioma therapy. *British journal of cancer* **95**, 869-878, doi:10.1038/sj.bjc.6603350 (2006).

254 Chen, H. W., Chang, Y. F., Chuang, H. Y., Tai, W. T. & Hwang, J. J. Targeted therapy with fatty acid synthase inhibitors in a human prostate carcinoma LNCaP/tk-luc-bearing animal model. *Prostate cancer and prostatic diseases* **15**, 260-264, doi:10.1038/pcan.2012.15 (2012).

255 Shah, U. S. *et al.* Fatty acid synthase gene overexpression and copy number gain in prostate adenocarcinoma. *Hum Pathol* **37**, 401-409, doi:10.1016/j.humpath.2005.11.022 (2006).

256 Gang, X. *et al.* P300 acetyltransferase regulates fatty acid synthase expression, lipid metabolism and prostate cancer growth. *Oncotarget* **7**, 15135-15149, doi:10.18632/oncotarget.7715 (2016).

257 Swinnen, J. V., Esquenet, M., Goossens, K., Heyns, W. & Verhoeven, G. Androgens stimulate fatty acid synthase in the human prostate cancer cell line LNCaP. *Cancer research* **57**, 1086-1090 (1997).

258 Swinnen, J. V. *et al.* Stimulation of tumor-associated fatty acid synthase expression by growth factor activation of the sterol regulatory element-binding protein pathway. *Oncogene* **19**, 5173-5181, doi:10.1038/sj.onc.1203889 (2000).

259 Huang, W. C., Li, X. Y., Liu, J., Lin, J. T. & Chung, L. W. K. Activation of Androgen Receptor, Lipogenesis, and Oxidative Stress Converged by SREBP-1 Is Responsible for Regulating Growth and Progression of Prostate Cancer Cells. *Mol Cancer Res* **10**, 133-142, doi:10.1158/1541-7786.MCR-11-0206 (2012).

260 Van de Sande, T. *et al.* High-level expression of fatty acid synthase in human prostate cancer tissues is linked to activation and nuclear localization of Akt/PKB. *J Pathol* **206**, 214-219, doi:10.1002/path.1760 (2005).

261 Van de Sande, T., De Schrijver, E., Heyns, W., Verhoeven, G. & Swinnen, J. V. Role of the phosphatidylinositol 3'-kinase/PTEN/Akt kinase pathway in the overexpression of fatty acid synthase in LNCaP prostate cancer cells. *Cancer research* **62**, 642-646 (2002).

262 Bandyopadhyay, S. *et al.* FAS expression inversely correlates with PTEN level in prostate cancer and a PI 3-kinase inhibitor synergizes with FAS siRNA to induce apoptosis. *Oncogene* **24**, 5389-5395, doi:10.1038/sj.onc.1208555 (2005).

263 Yang, Y. A., Han, W. F., Morin, P. J., Chrest, F. J. & Pizer, E. S. Activation of fatty acid synthesis during neoplastic transformation: role of mitogen-activated protein kinase and phosphatidylinositol 3-kinase. *Experimental cell research* **279**, 80-90 (2002).

264 Graner, E. *et al.* The isopeptidase USP2a regulates the stability of fatty acid synthase in prostate cancer. *Cancer cell* **5**, 253-261 (2004).

265 Ros, S. *et al.* Functional Metabolic Screen Identifies 6-Phosphofructo-2-Kinase/Fructose-2,6-Biphosphatase 4 as an Important Regulator of Prostate Cancer Cell Survival. *Cancer Discov* **2**, 328-343, doi:10.1158/2159-8290.CD-11-0234 (2012).

266 Shah, S. *et al.* Targeting ACLY sensitizes castration-resistant prostate cancer cells to AR antagonism by impinging on an ACLY-AMPK-AR feedback mechanism. *Oncotarget* **7**, 43713-43730, doi:10.18632/oncotarget.9666 (2016).

267 Moore, S. *et al.* Loss of stearoyl-CoA desaturase expression is a frequent event in prostate carcinoma. *Int J Cancer* **114**, 563-571, doi:10.1002/ijc.20773 (2005).

268 Fritz, V. *et al.* Abrogation of de novo lipogenesis by stearoyl-CoA desaturase 1 inhibition interferes with oncogenic signaling and blocks prostate cancer progression in mice. *Molecular cancer therapeutics* **9**, 1740-1754, doi:10.1158/1535-7163.MCT-09-1064 (2010).

269 Schaffer, J. E. Lipotoxicity: when tissues overeat. *Curr Opin Lipidol* **14**, 281-287, doi:10.1097/01.mol.0000073508.41685.7f (2003).

270 Kim, S. J., Choi, H., Park, S. S., Chang, C. S. & Kim, E. Stearoyl CoA desaturase (SCD) facilitates proliferation of prostate cancer cells through enhancement of androgen receptor transactivation. *Mol Cells* **31**, 371-377, doi:10.1007/s10059-011-0043-5 (2011).

271 Peck, B. *et al.* Inhibition of fatty acid desaturation is detrimental to cancer cell survival in metabolically compromised environments. *Cancer Metab* **4**, doi:Unsp 6

10.1186/S40170-016-0146-8 (2016).

272 Tamura, K. *et al.* Novel Lipogenic Enzyme ELOVL7 Is Involved in Prostate Cancer Growth through Saturated Long-Chain Fatty Acid Metabolism. *Cancer research* **69**, 8133-8140, doi:10.1158/0008-5472.CAN-09-0775 (2009).

273 Chen, Y. & Hughes-Fulford, M. Human prostate cancer cells lack feedback regulation of low-density lipoprotein receptor and its regulator, SREBP2. *Int J Cancer* **91**, 41-45 (2001).

274 Ettinger, S. L. *et al.* Dysregulation of sterol response element-binding proteins and downstream effectors in prostate cancer during progression to androgen independence. *Cancer research* **64**, 2212-2221 (2004).

275 Lee, B. H. *et al.* Dysregulation of Cholesterol Homeostasis in Human Prostate Cancer through Loss of ABCA1. *Cancer research* **73**, 1211-1218, doi:10.1158/0008-5472.CAN-12-3128 (2013).

276 Zhuang, L., Kim, J., Adam, R. M., Solomon, K. R. & Freeman, M. R. Cholesterol targeting alters lipid raft composition and cell survival in prostate cancer cells and xenografts. *The Journal of clinical investigation* **115**, 959-968, doi:10.1172/JCI19935 (2005).

277 Zhuang, L., Lin, J., Lu, M. L., Solomon, K. R. & Freeman, M. R. Cholesterol-rich lipid rafts mediate akt-regulated survival in prostate cancer cells. *Cancer research* **62**, 2227-2231 (2002).
278 Roy, M., Kung, H. J. & Ghosh, P. M. Statins and prostate cancer: role of cholesterol inhibition vs. prevention of small GTP-binding proteins. *American journal of cancer research* **1**, 542-561 (2011).
279 Sugie, S. *et al.* Significant Association of Caveolin-1 and Caveolin-2 with Prostate Cancer Progression. *Cancer genomics & proteomics* **12**, 391-396 (2015).
280 Freeman, M. R., Yang, W. & Di Vizio, D. Caveolin-1 and prostate cancer progression. *Advances in experimental medicine and biology* **729**, 95-110, doi:10.1007/978-1-4614-1222-9_7 (2012).
281 Bosch, M., Mari, M., Gross, S. P., Fernandez-Checa, J. C. & Pol, A. Mitochondrial Cholesterol: A Connection Between Caveolin, Metabolism, and Disease. *Traffic* **12**, 1483-1489, doi:10.1111/j.1600-0854.2011.01259.x (2011).
282 Lu, M. L., Schneider, M. C., Zheng, Y., Zhang, X. & Richie, J. P. Caveolin-1 interacts with androgen receptor. A positive modulator of androgen receptor mediated transactivation. *The Journal of biological chemistry* **276**, 13442-13451, doi:10.1074/jbc.M006598200 (2001).
283 Williams, T. M. *et al.* Caveolin-1 promotes tumor progression in an autochthonous mouse model of prostate cancer - Genetic ablation of Cav-1 delays advanced prostate tumor development in tramp mice. *Journal of Biological Chemistry* **280**, 25134-25145, doi:10.1074/jbc.M501186200 (2005).
284 Alfaqih, M. A., Allott, E. H., Hamilton, R. J., Freeman, M. R. & Freedland, S. J. The current evidence on statin use and prostate cancer prevention: are we there yet? *Nature Reviews Urology* **14**, 107-119, doi:10.1038/nrurol.2016.199 (2017).
285 Rubin, M. A. *et al.* alpha-methylacyl coenzyme A racemase as a tissue biomarker for prostate cancer. *Jama-J Am Med Assoc* **287**, 1662-1670, doi:DOI 10.1001/jama.287.13.1662 (2002).
286 Luo, J. *et al.* Alpha-methylacyl-CoA racemase: a new molecular marker for prostate cancer. *Cancer research* **62**, 2220-2226 (2002).
287 Jiang, N., Zhu, S., Chen, J., Niu, Y. & Zhou, L. A-methylacyl-CoA racemase (AMACR) and prostate-cancer risk: a meta-analysis of 4,385 participants. *PloS one* **8**, e74386, doi:10.1371/journal.pone.0074386 (2013).
288 Mobley, J. A. *et al.* Branched fatty acids in dairy and beef products markedly enhance alpha-methylacyl-CoA racemase expression in prostate cancer cells in vitro. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology* **12**, 775-783 (2003).
289 Zha, S. *et al.* Peroxisomal branched chain fatty acid beta-oxidation pathway is upregulated in prostate cancer. *The Prostate* **63**, 316-323, doi:10.1002/pros.20177 (2005).
290 Schrader, M. & Fahimi, H. D. Peroxisomes and oxidative stress. *Biochimica et biophysica acta* **1763**, 1755-1766, doi:10.1016/j.bbamcr.2006.09.006 (2006).
291 Ye, Z. W., Zhang, J., Townsend, D. M. & Tew, K. D. Oxidative stress, redox regulation and diseases of cellular differentiation. *Bba-Gen Subjects* **1850**, 1607-1621, doi:10.1016/j.bbagen.2014.11.010 (2015).
292 Schieber, M. & Chandel, N. S. ROS function in redox signaling and oxidative stress. *Current biology : CB* **24**, R453-462, doi:10.1016/j.cub.2014.03.034 (2014).
293 Holmstrom, K. M. & Finkel, T. Cellular mechanisms and physiological consequences of redox-dependent signalling. *Nature reviews. Molecular cell biology* **15**, 411-421, doi:10.1038/nrm3801 (2014).

294 Kalyanaraman, B. Teaching the basics of redox biology to medical and graduate students: Oxidants, antioxidants and disease mechanisms. *Redox biology* **1**, 244-257, doi:10.1016/j.redox.2013.01.014 (2013).
295 Guyton, K. Z. & Kensler, T. W. Oxidative mechanisms in carcinogenesis. *British medical bulletin* **49**, 523-544 (1993).
296 Cadet, J. & Wagner, J. R. DNA base damage by reactive oxygen species, oxidizing agents, and UV radiation. *Cold Spring Harbor perspectives in biology* **5**, doi:10.1101/cshperspect.a012559 (2013).
297 Klaunig, J. E., Kamendulis, L. M. & Hocevar, B. A. Oxidative stress and oxidative damage in carcinogenesis. *Toxicologic pathology* **38**, 96-109, doi:10.1177/0192623309356453 (2010).
298 Wei, H. Activation of oncogenes and/or inactivation of anti-oncogenes by reactive oxygen species. *Medical hypotheses* **39**, 267-270 (1992).
299 Cerutti, P. A. Oxy-radicals and cancer. *Lancet* **344**, 862-863 (1994).
300 Halliwell, B. Free radicals, antioxidants, and human disease: curiosity, cause, or consequence? *Lancet* **344**, 721-724 (1994).
301 Ouyang, X., DeWeese, T. L., Nelson, W. G. & Abate-Shen, C. Loss-of-function of Nkx3.1 promotes increased oxidative damage in prostate carcinogenesis. *Cancer research* **65**, 6773-6779, doi:10.1158/0008-5472.CAN-05-1948 (2005).
302 Tam, N. N. *et al.* Differential attenuation of oxidative/nitrosative injuries in early prostatic neoplastic lesions in TRAMP mice by dietary antioxidants. *The Prostate* **66**, 57-69, doi:10.1002/pros.20313 (2006).
303 Tam, N. N., Leav, I. & Ho, S. M. Sex hormones induce direct epithelial and inflammation-mediated oxidative/nitrosative stress that favors prostatic carcinogenesis in the noble rat. *The American journal of pathology* **171**, 1334-1341, doi:10.2353/ajpath.2007.070199 (2007).
304 Kumar, B., Koul, S., Khandrika, L., Meacham, R. B. & Koul, H. K. Oxidative stress is inherent in prostate cancer cells and is required for aggressive phenotype. *Cancer research* **68**, 1777-1785, doi:10.1158/0008-5472.CAN-07-5259 (2008).
305 Turpaev, K. T. Reactive oxygen species and regulation of gene expression. *Biochemistry. Biokhimiia* **67**, 281-292 (2002).
306 Fleshner, N. E. & Klotz, L. H. Diet, androgens, oxidative stress and prostate cancer susceptibility. *Cancer metastasis reviews* **17**, 325-330 (1998).
307 Tam, N. N., Gao, Y., Leung, Y. K. & Ho, S. M. Androgenic regulation of oxidative stress in the rat prostate: involvement of NAD(P)H oxidases and antioxidant defense machinery during prostatic involution and regrowth. *The American journal of pathology* **163**, 2513-2522, doi:10.1016/S0002-9440(10)63606-1 (2003).
308 Lin, X. *et al.* Reversal of GSTP1 CpG island hypermethylation and reactivation of pi-class glutathione S-transferase (GSTP1) expression in human prostate cancer cells by treatment with procainamide. *Cancer research* **61**, 8611-8616 (2001).
309 Bostwick, D. G. *et al.* Antioxidant enzyme expression and reactive oxygen species damage in prostatic intraepithelial neoplasia and cancer. *Cancer* **89**, 123-134 (2000).
310 Oberley, T. D., Zhong, W., Szweda, L. I. & Oberley, L. W. Localization of antioxidant enzymes and oxidative damage products in normal and malignant prostate epithelium. *The Prostate* **44**, 144-155 (2000).

311 Yossepowitch, O. *et al.* Advanced but not localized prostate cancer is associated with increased oxidative stress. *The Journal of urology* **178**, 1238-1243; discussion 1243-1234, doi:10.1016/j.juro.2007.05.145 (2007).
312 Aydin, A. *et al.* Oxidative stress and antioxidant status in non-metastatic prostate cancer and benign prostatic hyperplasia. *Clinical biochemistry* **39**, 176-179, doi:10.1016/j.clinbiochem.2005.11.018 (2006).
313 Arsova-Sarafinovska, Z. *et al.* Increased oxidative/nitrosative stress and decreased antioxidant enzyme activities in prostate cancer. *Clinical biochemistry* **42**, 1228-1235, doi:10.1016/j.clinbiochem.2009.05.009 (2009).
314 Kaya, E. *et al.* Oxidative stress parameters in patients with prostate cancer, benign prostatic hyperplasia and asymptomatic inflammatory prostatitis: A prospective controlled study. *Advances in clinical and experimental medicine : official organ Wroclaw Medical University* **26**, 1095-1099, doi:10.17219/acem/66837 (2017).
315 Carter, H. B. *et al.* Early detection of prostate cancer: AUA Guideline. *The Journal of urology* **190**, 419-426, doi:10.1016/j.juro.2013.04.119 (2013).
316 Chodak, G. W. & Schoenberg, H. W. Early detection of prostate cancer by routine screening. *Jama* **252**, 3261-3264 (1984).
317 Alberts, A. R., Schoots, I. G. & Roobol, M. J. Prostate-specific antigen-based prostate cancer screening: Past and future. *Int J Urol* **22**, 524-532, doi:10.1111/iju.12750 (2015).
318 Hayes, J. H. & Barry, M. J. Screening for prostate cancer with the prostate-specific antigen test: a review of current evidence. *Jama* **311**, 1143-1149, doi:10.1001/jama.2014.2085 (2014).
319 Etzioni, R., Gulati, R., Falcon, S. & Penson, D. F. Impact of PSA screening on the incidence of advanced stage prostate cancer in the United States: a surveillance modeling approach. *Medical decision making : an international journal of the Society for Medical Decision Making* **28**, 323-331, doi:10.1177/0272989X07312719 (2008).
320 Barry, M. J. Screening for Prostate Cancer - The Controversy That Refuses to Die. *New Engl J Med* **360**, 1351-1354, doi:10.1056/Nejme0901166 (2009).
321 Andriole, G. L. *et al.* Prostate cancer screening in the randomized Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial: mortality results after 13 years of follow-up. *Journal of the National Cancer Institute* **104**, 125-132, doi:10.1093/jnci/djr500 (2012).
322 Heijnsdijk, E. A. *et al.* Quality-of-life effects of prostate-specific antigen screening. *The New England journal of medicine* **367**, 595-605, doi:10.1056/NEJMoa1201637 (2012).
323 Ilic, D., Neuberger, M. M., Djulbegovic, M. & Dahm, P. Screening for prostate cancer. *The Cochrane database of systematic reviews*, CD004720, doi:10.1002/14651858.CD004720.pub3 (2013).
324 Schroder, F. H. *et al.* Screening and prostate cancer mortality: results of the European Randomised Study of Screening for Prostate Cancer (ERSPC) at 13 years of follow-up. *Lancet* **384**, 2027-2035, doi:10.1016/S0140-6736(14)60525-0 (2014).
325 Gjertson, C. K. & Albertsen, P. C. Use and Assessment of PSA in Prostate Cancer. *Med Clin N Am* **95**, 191-+, doi:10.1016/j.mcna.2010.08.024 (2011).
326 Greene, K. L. *et al.* Prostate specific antigen best practice statement: 2009 update. *The Journal of urology* **182**, 2232-2241, doi:10.1016/j.juro.2009.07.093 (2009).
327 Schroder, F. H. *et al.* Screening and prostate-cancer mortality in a randomized European study. *The New England journal of medicine* **360**, 1320-1328, doi:10.1056/NEJMoa0810084 (2009).

328 Heijnsdijk, E. A. *et al.* Overdetection, overtreatment and costs in prostate-specific antigen screening for prostate cancer. *British journal of cancer* **101**, 1833-1838, doi:10.1038/sj.bjc.6605422 (2009).
329 Duffy, M. J. Prostate-specific antigen: does the current evidence support its use in prostate cancer screening? *Annals of clinical biochemistry* **48**, 310-316, doi:10.1258/acb.2011.010273 (2011).
330 Carter, H. B. Prostate cancers in men with low PSA levels - Must we find them? *New Engl J Med* **350**, 2292-2294, doi:Doi 10.1056/Nejme048003 (2004).
331 Dahm, P. Future of screening for prostate cancer. *Bmj* **358**, j4200, doi:10.1136/bmj.j4200 (2017).
332 Futterer, J. J. *et al.* Can Clinically Significant Prostate Cancer Be Detected with Multiparametric Magnetic Resonance Imaging? A Systematic Review of the Literature. *Eur Urol* **68**, 1045-1053, doi:10.1016/j.eururo.2015.01.013 (2015).
333 Ukimura, O. *et al.* Contemporary role of systematic prostate biopsies: indications, techniques, and implications for patient care. *Eur Urol* **63**, 214-230, doi:10.1016/j.eururo.2012.09.033 (2013).
334 Bjurlin, M. A. *et al.* Optimization of initial prostate biopsy in clinical practice: sampling, labeling and specimen processing. *The Journal of urology* **189**, 2039-2046, doi:10.1016/j.juro.2013.02.072 (2013).
335 Iczkowski, K. A. & Lucia, M. S. Current perspectives on Gleason grading of prostate cancer. *Current urology reports* **12**, 216-222, doi:10.1007/s11934-011-0181-5 (2011).
336 Heidenreich, A. *et al.* EAU Guidelines on Prostate Cancer. Part 1: Screening, Diagnosis, and Local Treatment with Curative Intent-Update 2013. *Eur Urol* **65**, 124-137, doi:10.1016/j.eururo.2013.09.046 (2014).
337 Network, N. C. C. *NCCN Clinical Practice Guidelines in Oncology Prostate Cancer Version 3.2016* 2016).
338 Garisto, J. D. & Klotz, L. Active Surveillance for Prostate Cancer: How to Do It Right. *Oncology-Ny* **31**, 333-340 (2017).
339 Lancee, M. *et al.* Guideline of guidelines: primary monotherapies for localised or locally advanced prostate cancer. *BJU international*, doi:10.1111/bju.14237 (2018).
340 Miller, K. D. *et al.* Cancer treatment and survivorship statistics, 2016. *CA: a cancer journal for clinicians* **66**, 271-289, doi:10.3322/caac.21349 (2016).
341 Potosky, A. L. *et al.* Health outcomes after prostatectomy or radiotherapy for prostate cancer: results from the Prostate Cancer Outcomes Study. *Journal of the National Cancer Institute* **92**, 1582-1592 (2000).
342 Stanford, J. L. *et al.* Urinary and sexual function after radical prostatectomy for clinically localized prostate cancer: the Prostate Cancer Outcomes Study. *Jama* **283**, 354-360 (2000).
343 Walz, J. *et al.* A Critical Analysis of the Current Knowledge of Surgical Anatomy of the Prostate Related to Optimisation of Cancer Control and Preservation of Continence and Erection in Candidates for Radical Prostatectomy: An Update. *Eur Urol* **70**, 301-311, doi:10.1016/j.eururo.2016.01.026 (2016).
344 Lee, T., Mendhiratta, N., Sperling, D. & Lepor, H. Focal laser ablation for localized prostate cancer: principles, clinical trials, and our initial experience. *Reviews in urology* **16**, 55-66 (2014).
345 Huggins, C. Endocrine-induced regression of cancers. *Cancer research* **27**, 1925-1930 (1967).

346 Eisenberger, M. A. *et al.* Bilateral orchiectomy with or without flutamide for metastatic prostate cancer. *The New England journal of medicine* **339**, 1036-1042, doi:10.1056/NEJM199810083391504 (1998).

347 O'Hara, L. *et al.* Pituitary Androgen Receptor Signalling Regulates Prolactin but Not Gonadotrophins in the Male Mouse. *PloS one* **10**, doi:UNSP e0121657

10.1371/journal.pone.0121657 (2015).

348 Ammannagari, N. & George, S. Anti-Androgen Therapies for Prostate Cancer: A Focused Review. *Am J Hematol-Oncol* **11**, 15-19 (2015).

349 Paller, C. J. & Antonarakis, E. S. Management of biochemically recurrent prostate cancer after local therapy: evolving standards of care and new directions. *Clinical advances in hematology & oncology : H&O* **11**, 14-23 (2013).

350 Roehl, K. A., Han, M., Ramos, C. G., Antenor, J. A. & Catalona, W. J. Cancer progression and survival rates following anatomical radical retropubic prostatectomy in 3,478 consecutive patients: long-term results. *The Journal of urology* **172**, 910-914, doi:10.1097/01.ju.0000134888.22332.bb (2004).

351 Freedland, S. J. *et al.* Risk of prostate cancer-specific mortality following biochemical recurrence after radical prostatectomy. *Jama* **294**, 433-439, doi:10.1001/jama.294.4.433 (2005).

352 Kupelian, P. A., Mahadevan, A., Reddy, C. A., Reuther, A. M. & Klein, E. A. Use of different definitions of biochemical failure after external beam radiotherapy changes conclusions about relative treatment efficacy for localized prostate cancer. *Urology* **68**, 593-598, doi:10.1016/j.urology.2006.03.075 (2006).

353 Kolodziej, M. Management of biochemically recurrent prostate cancer following local therapy. *The American journal of managed care* **20**, S273-281 (2014).

354 Ritch, C. R. & Cookson, M. S. Advances in the management of castration resistant prostate cancer. *Bmj* **355**, i4405, doi:10.1136/bmj.i4405 (2016).

355 Tran, C. *et al.* Development of a second-generation antiandrogen for treatment of advanced prostate cancer. *Science* **324**, 787-790, doi:10.1126/science.1168175 (2009).

356 Hammerstrom, A. E., Cauley, D. H., Atkinson, B. J. & Sharma, P. Cancer immunotherapy: sipuleucel-T and beyond. *Pharmacotherapy* **31**, 813-828, doi:10.1592/phco.31.8.813 (2011).

357 Fizazi, K. *et al.* Abiraterone acetate for treatment of metastatic castration-resistant prostate cancer: final overall survival analysis of the COU-AA-301 randomised, double-blind, placebo-controlled phase 3 study. *The Lancet. Oncology* **13**, 983-992, doi:10.1016/S1470-2045(12)70379-0 (2012).

358 Ryan, C. J. *et al.* Abiraterone acetate plus prednisone versus placebo plus prednisone in chemotherapy-naive men with metastatic castration-resistant prostate cancer (COU-AA-302): final overall survival analysis of a randomised, double-blind, placebo-controlled phase 3 study. *The Lancet. Oncology* **16**, 152-160, doi:10.1016/S1470-2045(14)71205-7 (2015).

359 Scher, H. I. *et al.* Increased survival with enzalutamide in prostate cancer after chemotherapy. *The New England journal of medicine* **367**, 1187-1197, doi:10.1056/NEJMoa1207506 (2012).

360 Kantoff, P. W. *et al.* Sipuleucel-T immunotherapy for castration-resistant prostate cancer. *The New England journal of medicine* **363**, 411-422, doi:10.1056/NEJMoa1001294 (2010).

361 Berthold, D. R. *et al.* Docetaxel plus prednisone or mitoxantrone plus prednisone for advanced prostate cancer: updated survival in the TAX 327 study. *Journal of clinical oncology : official*

journal of the American Society of Clinical Oncology **26**, 242-245, doi:10.1200/JCO.2007.12.4008 (2008).

362 de Bono, J. S. *et al.* Prednisone plus cabazitaxel or mitoxantrone for metastatic castration-resistant prostate cancer progressing after docetaxel treatment: a randomised open-label trial. *Lancet* **376**, 1147-1154, doi:10.1016/S0140-6736(10)61389-X (2010).

363 Parker, C. *et al.* Updated analysis of the phase III, double-blind, randomized, multinational study of radium-223 chloride in castration-resistant prostate cancer (CRPC) patients with bone metastases (ALSYMPCA). *Journal of Clinical Oncology* **30**, doi:DOI 10.1200/jco.2012.30.18_suppl.lba4512 (2012).

364 Sporn, M. B., Dunlop, N. M., Newton, D. L. & Smith, J. M. Prevention of chemical carcinogenesis by vitamin A and its synthetic analogs (retinoids). *Federation proceedings* **35**, 1332-1338 (1976).

365 Sakr, W. A. & Partin, A. W. Histological markers of risk and the role of high-grade prostatic intraepithelial neoplasia. *Urology* **57**, 115-120 (2001).

366 Dunn, B. K., Umar, A. & Richmond, E. Introduction: Cancer chemoprevention and its context. *Seminars in oncology* **43**, 19-21, doi:10.1053/j.seminoncol.2015.11.002 (2016).

367 Al Rabadi, L. & Bergan, R. A Way Forward for Cancer Chemoprevention: Think Local. *Cancer prevention research* **10**, 14-35, doi:10.1158/1940-6207.CAPR-16-0194 (2017).

368 Liu, Y. Q. *et al.* Prostate cancer chemoprevention agents exhibit selective activity against early stage prostate cancer cells. *Prostate cancer and prostatic diseases* **4**, 81-91, doi:10.1038/sj.pcan.4500506 (2001).

369 Roehrborn, C. G. & Black, L. K. The economic burden of prostate cancer. *BJU international* **108**, 806-813, doi:10.1111/j.1464-410X.2011.10365.x (2011).

370 Steele V.E., L. R. A., Moon R.C. . *Preclinical Animal Models for the Development of Cancer Chemoprevention Drugs*. Vol. 2 (Humana Press, 2005).

371 Ittmann, M. *et al.* Animal models of human prostate cancer: the consensus report of the New York meeting of the Mouse Models of Human Cancers Consortium Prostate Pathology Committee. *Cancer research* **73**, 2718-2736, doi:10.1158/0008-5472.CAN-12-4213 (2013).

372 Grabowska, M. M. *et al.* Mouse models of prostate cancer: picking the best model for the question. *Cancer metastasis reviews* **33**, 377-397, doi:10.1007/s10555-013-9487-8 (2014).

373 Greenberg, N. M. *et al.* Prostate cancer in a transgenic mouse. *Proceedings of the National Academy of Sciences of the United States of America* **92**, 3439-3443 (1995).

374 Gingrich, J. R. *et al.* Metastatic prostate cancer in a transgenic mouse. *Cancer research* **56**, 4096-4102 (1996).

375 Kaplan-Lefko, P. J. *et al.* Pathobiology of autochthonous prostate cancer in a pre-clinical transgenic mouse model. *The Prostate* **55**, 219-237, doi:10.1002/pros.10215 (2003).

376 Gingrich, J. R. *et al.* Androgen-independent prostate cancer progression in the TRAMP model. *Cancer research* **57**, 4687-4691 (1997).

377 Kasper, S. *et al.* Development, progression, and androgen-dependence of prostate tumors in probasin-large T antigen transgenic mice: a model for prostate cancer. *Lab Invest* **78**, i-xv (1998).

378 Koike, H. *et al.* Conditional PTEN-deficient mice as a prostate cancer chemoprevention model. *Asian Pacific journal of cancer prevention : APJCP* **16**, 1827-1831 (2015).

379 Banach-Petrosky, W. *et al.* Vitamin D inhibits the formation of prostatic intraepithelial neoplasia in Nkx3.1;Pten mutant mice. *Clin Cancer Res* **12**, 5895-5901, doi:10.1158/1078-0432.CCR-06-1039 (2006).

380 Abate-Shen, C. *et al.* Nkx3.1; Pten mutant mice develop invasive prostate adenocarcinoma and lymph node metastases. *Cancer research* **63**, 3886-3890 (2003).
381 Parimi, V., Goyal, R., Poropatich, K. & Yang, X. J. Neuroendocrine differentiation of prostate cancer: a review. *American journal of clinical and experimental urology* **2**, 273-285 (2014).
382 Barbieri, C. E. *et al.* The mutational landscape of prostate cancer. *Eur Urol* **64**, 567-576, doi:10.1016/j.eururo.2013.05.029 (2013).
383 Lamb, D. J. & Zhang, L. Challenges in prostate cancer research: animal models for nutritional studies of chemoprevention and disease progression. *J Nutr* **135**, 3009S-3015S (2005).
384 Dunning, W. F. Prostate Cancer in the Rat. *National Cancer Institute monograph* **12**, 351-369 (1963).
385 McCormick, D. L. *et al.* Chemoprevention of rat prostate carcinogenesis by dietary 16 alpha-fluoro-5-androsten-17-one (fluasterone), a minimally androgenic analog of dehydroepiandrosterone. *Carcinogenesis* **28**, 398-403, doi:10.1093/carcin/bgl141 (2007).
386 Bosland, M. C., Prinsen, M. K. & Kroes, R. Adenocarcinomas of the prostate induced by N-nitroso-N-methylurea in rats pretreated with cyproterone acetate and testosterone. *Cancer letters* **18**, 69-78 (1983).
387 Andriole, G. L. *et al.* Effect of dutasteride on the risk of prostate cancer. *The New England journal of medicine* **362**, 1192-1202, doi:10.1056/NEJMoa0908127 (2010).
388 Thompson, I. M. *et al.* The influence of finasteride on the development of prostate cancer. *The New England journal of medicine* **349**, 215-224, doi:10.1056/NEJMoa030660 (2003).
389 Hamilton, R. J. & Freedland, S. J. 5-alpha reductase inhibitors and prostate cancer prevention: where do we turn now? *Bmc Med* **9**, doi:Artn 105

10.1186/1741-7015-9-105 (2011).
390 Brigelius-Flohe, R. & Traber, M. G. Vitamin E: function and metabolism. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **13**, 1145-1155 (1999).
391 Joffe, M. & Harris, P. L. The biological potency of the natural tocopherols and certain derivatives. *J Am Chem Soc* **65**, 925-927, doi:Doi 10.1021/Ja01245a042 (1943).
392 Harris, P. L. & Ludwig, M. I. Relative vitamin E potency of natural and of synthetic alpha-tocopherol. *The Journal of biological chemistry* **179**, 1111-1115 (1949).
393 Lee, E. *et al.* Alpha-tocopheryl succinate, in contrast to alpha-tocopherol and alpha-tocopheryl acetate, inhibits prostaglandin E2 production in human lung epithelial cells. *Carcinogenesis* **27**, 2308-2315, doi:10.1093/carcin/bgl073 (2006).
394 Cooney, R. V. *et al.* Gamma-tocopherol detoxification of nitrogen dioxide: superiority to alpha-tocopherol. *Proceedings of the National Academy of Sciences of the United States of America* **90**, 1771-1775 (1993).
395 Ju, J. *et al.* Cancer-preventive activities of tocopherols and tocotrienols. *Carcinogenesis* **31**, 533-542, doi:10.1093/carcin/bgp205 (2010).
396 Constantinou, C., Papas, A. & Constantinou, A. I. Vitamin E and cancer: An insight into the anticancer activities of vitamin E isomers and analogs. *Int J Cancer* **123**, 739-752, doi:10.1002/ijc.23689 (2008).
397 Gunawardena, K., Murray, D. K. & Meikle, A. W. Vitamin E and other antioxidants inhibit human prostate cancer cells through apoptosis. *The Prostate* **44**, 287-295 (2000).

398 Crispen, P. L. *et al.* Vitamin E succinate inhibits NF-kappa B and prevents the development of a metastatic phenotype in prostate cancer cells: Implications for chemoprevention. *The Prostate* **67**, 582-590, doi:10.1002/pros.20468 (2007).

399 Shiau, C. W. *et al.* alpha-Tocopheryl succinate induces apoptosis in prostate cancer cells in part through inhibition of Bcl-xL/Bcl-2 function. *The Journal of biological chemistry* **281**, 11819-11825, doi:10.1074/jbc.M511015200 (2006).

400 Ni, J. *et al.* Vitamin E succinate inhibits human prostate cancer cell growth via modulating cell cycle regulatory machinery. *Biochemical and biophysical research communications* **300**, 357-363 (2003).

401 Morley, S. *et al.* Tocopherol transfer protein sensitizes prostate cancer cells to vitamin E. *The Journal of biological chemistry* **285**, 35578-35589, doi:10.1074/jbc.M110.169664 (2010).

402 Arita, M. *et al.* Human alpha-tocopherol transfer protein: cDNA cloning, expression and chromosomal localization. *The Biochemical journal* **306 (Pt 2)**, 437-443 (1995).

403 Chang, E. *et al.* Alpha-vitamin E derivative, RRR-alpha-tocopheryloxybutyric acid inhibits the proliferation of prostate cancer cells. *Asian journal of andrology* **9**, 31-39, doi:10.1111/j.1745-7262.2007.00246.x (2007).

404 Zhang, Y. *et al.* Vitamin E succinate inhibits the function of androgen receptor and the expression of prostate-specific antigen in prostate cancer cells. *Proceedings of the National Academy of Sciences of the United States of America* **99**, 7408-7413, doi:10.1073/pnas.102014399 (2002).

405 Israel, K., Yu, W., Sanders, B. G. & Kline, K. Vitamin E succinate induces apoptosis in human prostate cancer cells: role for Fas in vitamin E succinate-triggered apoptosis. *Nutrition and cancer* **36**, 90-100, doi:10.1207/S15327914NC3601_13 (2000).

406 Jia, L. *et al.* Critical roles for JNK, c-Jun, and Fas/FasL-Signaling in vitamin E analog-induced apoptosis in human prostate cancer cells. *The Prostate* **68**, 427-441, doi:10.1002/pros.20716 (2008).

407 Yin, Y. *et al.* The therapeutic and preventive effect of RRR-alpha-vitamin E succinate on prostate cancer via induction of insulin-like growth factor binding protein-3. *Clin Cancer Res* **13**, 2271-2280, doi:10.1158/1078-0432.CCR-06-1217 (2007).

408 Jensen, S. K., Engberg, R. M. & Hedemann, M. S. All-rac-alpha-tocopherol acetate is a better vitamin E source than all-rac-alpha-tocopherol succinate for broilers. *J Nutr* **129**, 1355-1360 (1999).

409 Ni, J. *et al.* In vitro and in vivo anticancer effects of the novel vitamin E ether analogue RRR-alpha-tocopheryloxybutyl sulfonic acid in prostate cancer. *Clin Cancer Res* **15**, 898-906, doi:10.1158/1078-0432.CCR-08-1087 (2009).

410 Fleshner, N., Fair, W. R., Huryk, R. & Heston, W. D. Vitamin E inhibits the high-fat diet promoted growth of established human prostate LNCaP tumors in nude mice. *The Journal of urology* **161**, 1651-1654 (1999).

411 Basu, A., Grossie, B., Bennett, M., Mills, N. & Imrhan, V. Alpha-tocopheryl succinate (alpha-TOS) modulates human prostate LNCaP xenograft growth and gene expression in BALB/c nude mice fed two levels of dietary soybean oil. *European journal of nutrition* **46**, 34-43, doi:10.1007/s00394-006-0629-4 (2007).

412 Takahashi, S. *et al.* Suppression of prostate cancer in a transgenic rat model via gamma-tocopherol activation of caspase signaling. *The Prostate* **69**, 644-651, doi:10.1002/pros.20915 (2009).

413 Barve, A. *et al.* Gamma-tocopherol-enriched mixed tocopherol diet inhibits prostate carcinogenesis in TRAMP mice. *Int J Cancer* **124**, 1693-1699, doi:10.1002/ijc.24106 (2009).
414 Chen, J. X. *et al.* Dietary tocopherols inhibit PhIP-induced prostate carcinogenesis in CYP1A-humanized mice. *Cancer letters* **371**, 71-78, doi:10.1016/j.canlet.2015.11.010 (2016).
415 Huang, P. H. *et al.* Vitamin E facilitates the inactivation of the kinase Akt by the phosphatase PHLPP1. *Science signaling* **6**, ra19, doi:10.1126/scisignal.2003816 (2013).
416 Jiang, Q., Wong, J., Fyrst, H., Saba, J. D. & Ames, B. N. gamma-Tocopherol or combinations of vitamin E forms induce cell death in human prostate cancer cells by interrupting sphingolipid synthesis. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 17825-17830, doi:10.1073/pnas.0408340102 (2004).
417 Huang, H. R. *et al.* Potent Inhibitory Effect of delta-Tocopherol on Prostate Cancer Cells Cultured in Vitro and Grown As Xenograft Tumors in Vivo. *J Agr Food Chem* **62**, 10752-10758, doi:10.1021/jf504058f (2014).
418 Wang, H. *et al.* delta-Tocopherol inhibits the development of prostate adenocarcinoma in prostate specific Pten-/- mice. *Carcinogenesis* **39**, 158-169, doi:10.1093/carcin/bgx128 (2018).
419 Sanches, L. D. *et al.* Protective effect of gamma-tocopherol-enriched diet on N-methyl-N-nitrosourea-induced epithelial dysplasia in rat ventral prostate. *Int J Exp Pathol* **94**, 362-372, doi:10.1111/iep.12042 (2013).
420 Cardoso, B. R., Roberts, B. R., Bush, A. I. & Hare, D. J. Selenium, selenoproteins and neurodegenerative diseases. *Metallomics : integrated biometal science* **7**, 1213-1228, doi:10.1039/c5mt00075k (2015).
421 Labunskyy, V. M., Hatfield, D. L. & Gladyshev, V. N. Selenoproteins: molecular pathways and physiological roles. *Physiological reviews* **94**, 739-777, doi:10.1152/physrev.00039.2013 (2014).
422 Zhong, W. & Oberley, T. D. Redox-mediated effects of selenium on apoptosis and cell cycle in the LNCaP human prostate cancer cell line. *Cancer research* **61**, 7071-7078 (2001).
423 Webber, M. M., Perez-Ripoll, E. A. & James, G. T. Inhibitory effects of selenium on the growth of DU-145 human prostate carcinoma cells in vitro. *Biochemical and biophysical research communications* **130**, 603-609 (1985).
424 He, Q. *et al.* Death receptor 5 regulation during selenium-mediated apoptosis in human prostate cancer cells. *Cancer biology & therapy* **1**, 287-290 (2002).
425 Redman, C. *et al.* Inhibitory effect of selenomethionine on the growth of three selected human tumor cell lines. *Cancer letters* **125**, 103-110 (1998).
426 Gundimeda, U. *et al.* Locally generated methylseleninic acid induces specific inactivation of protein kinase C isoenzymes: relevance to selenium-induced apoptosis in prostate cancer cells. *The Journal of biological chemistry* **283**, 34519-34531, doi:10.1074/jbc.M807007200 (2008).
427 Gundimeda, U., Schiffman, J. E., Gottlieb, S. N., Roth, B. I. & Gopalakrishna, R. Negation of the cancer-preventive actions of selenium by over-expression of protein kinase Cepsilon and selenoprotein thioredoxin reductase. *Carcinogenesis* **30**, 1553-1561, doi:10.1093/carcin/bgp164 (2009).
428 Gasparian, A. V. *et al.* Selenium compounds inhibit I kappa B kinase (IKK) and nuclear factor-kappa B (NF-kappa B) in prostate cancer cells. *Molecular cancer therapeutics* **1**, 1079-1087 (2002).
429 Christensen, M. J., Nartey, E. T., Hada, A. L., Legg, R. L. & Barzee, B. R. High selenium reduces NF-kappaB-regulated gene expression in uninduced human prostate cancer cells. *Nutrition and cancer* **58**, 197-204, doi:10.1080/01635580701328701 (2007).

430 Lu, Z. *et al.* Se-methylselenocysteine suppresses the growth of prostate cancer cell DU145 through connexin 43-induced apoptosis. *J Cancer Res Ther* **11**, 840-845, doi:10.4103/0973-1482.139265 (2015).

431 Jiang, C., Wang, Z., Ganther, H. & Lu, J. X. Caspases as key executors of methyl selenium-induced apoptosis (Anoikis) of DU-145 prostate cancer cells. *Cancer research* **61**, 3062-3070 (2001).

432 Menter, D. G., Sabichi, A. L. & Lippman, S. M. Selenium effects on prostate cell growth. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology* **9**, 1171-1182 (2000).

433 Jiang, C., Wang, Z., Ganther, H. & Lu, J. Distinct effects of methylseleninic acid versus selenite on apoptosis, cell cycle, and protein kinase pathways in DU145 human prostate cancer cells. *Molecular cancer therapeutics* **1**, 1059-1066 (2002).

434 Wang, Z., Jiang, C. & Lu, J. Induction of caspase-mediated apoptosis and cell-cycle G1 arrest by selenium metabolite methylselenol. *Molecular carcinogenesis* **34**, 113-120, doi:10.1002/mc.10056 (2002).

435 Liu, S. A., Zhang, H. T., Zhu, L. Y., Zhao, L. J. & Dong, Y. Kruppel-like factor 4 is a novel mediator of selenium in growth inhibition. *Mol Cancer Res* **6**, 306-313, doi:10.1158/1541-7786.MCR-07-0159 (2008).

436 Venkateswaran, V., Klotz, L. H. & Fleshner, N. E. Selenium modulation of cell proliferation and cell cycle biomarkers in human prostate carcinoma cell lines. *Cancer research* **62**, 2540-2545 (2002).

437 Dong, Y. *et al.* Prostate specific antigen expression is down-regulated by selenium through disruption of androgen receptor signaling. *Cancer research* **64**, 19-22, doi:Doi 10.1158/0008-5472.Can-03-2789 (2004).

438 Cho, S. D. *et al.* Methyl selenium metabolites decrease prostate-specific antigen expression by inducing protein degradation and suppressing androgen-stimulated transcription. *Molecular cancer therapeutics* **3**, 605-611 (2004).

439 Dong, Y., Zhang, H. T., Gao, A. C., Marshall, J. R. & Ip, C. Androgen receptor signaling intensity is a key factor in determining the sensitivity of prostate cancer cells to selenium inhibition of growth and cancer-specific biomarkers. *Molecular cancer therapeutics* **4**, 1047-1055, doi:Doi 10.1158/1535-7163.Mct-05-0124 (2005).

440 Chun, J. Y. *et al.* Mechanisms of selenium down-regulation of androgen receptor signaling in prostate cancer. *Molecular cancer therapeutics* **5**, 913-918, doi:10.1158/1535-7163.MCT-05-0389 (2006).

441 Husbeck, B., Bhattacharyya, R. S., Feldman, D. & Knox, S. J. Inhibition of androgen receptor signaling by selenite and methylseleninic acid in prostate cancer cells: two distinct mechanisms of action. *Molecular cancer therapeutics* **5**, 2078-2085, doi:10.1158/1535-7163.MCT-06-0056 (2006).

442 Gazi, M. H., Gong, A., Donkena, K. V. & Young, C. Y. Sodium selenite inhibits interleukin-6-mediated androgen receptor activation in prostate cancer cells via upregulation of c-Jun. *Clinica chimica acta; international journal of clinical chemistry* **380**, 145-150, doi:10.1016/j.cca.2007.01.031 (2007).

443 Morris, J. D. *et al.* Selenium- or quercetin-induced retardation of DNA synthesis in primary prostate cells occurs in the presence of a concomitant reduction in androgen-receptor activity. *Cancer letters* **239**, 111-122, doi:10.1016/j.canlet.2005.07.037 (2006).

444 Ghosh, J. Rapid induction of apoptosis in prostate cancer cells by selenium: reversal by metabolites of arachidonate 5-lipoxygenase. *Biochemical and biophysical research communications* **315**, 624-635, doi:10.1016/j.bbrc.2004.01.100 (2004).

445 Jiang, C., Hu, H., Malewicz, B., Wang, Z. & Lu, J. Selenite-induced p53 Ser-15 phosphorylation and caspase-mediated apoptosis in LNCaP human prostate cancer cells. *Molecular cancer therapeutics* **3**, 877-884 (2004).

446 Ghosh, J. & Myers, C. E. Arachidonic acid stimulates prostate cancer cell growth: critical role of 5-lipoxygenase. *Biochemical and biophysical research communications* **235**, 418-423, doi:10.1006/bbrc.1997.6799 (1997).

447 Chaudry, A. A., Wahle, K. W., McClinton, S. & Moffat, L. E. Arachidonic acid metabolism in benign and malignant prostatic tissue in vitro: effects of fatty acids and cyclooxygenase inhibitors. *Int J Cancer* **57**, 176-180 (1994).

448 Anderson, K. M., Wygodny, J. B., Ondrey, F. & Harris, J. Human Pc-3 Prostate Cell-Line DNA-Synthesis Is Suppressed by Eicosatetraynoic Acid, an Invitro Inhibitor of Arachidonic-Acid Metabolism. *The Prostate* **12**, 3-12, doi:DOI 10.1002/pros.2990120103 (1988).

449 Hu, H., Jiang, C., Li, G. & Lu, J. PKB/AKT and ERK regulation of caspase-mediated apoptosis by methylseleninic acid in LNCaP prostate cancer cells. *Carcinogenesis* **26**, 1374-1381, doi:10.1093/carcin/bgi094 (2005).

450 Zu, K. *et al.* Enhanced selenium effect on growth arrest by BiP/GRP78 knockdown in p53-null human prostate cancer cells. *Oncogene* **25**, 546-554, doi:10.1038/sj.onc.1209071 (2006).

451 Wu, Y., Zhang, H., Dong, Y., Park, Y. M. & Ip, C. Endoplasmic reticulum stress signal mediators are targets of selenium action. *Cancer research* **65**, 9073-9079, doi:10.1158/0008-5472.CAN-05-2016 (2005).

452 Husbeck, B., Nonn, L., Peehl, D. M. & Knox, S. J. Tumor-selective killing by selenite in patient-matched pairs of normal and malignant prostate cells. *The Prostate* **66**, 218-225, doi:10.1002/pros.20337 (2006).

453 Zhao, R., Xiang, N., Domann, F. E. & Zhong, W. Expression of p53 enhances selenite-induced superoxide production and apoptosis in human prostate cancer cells. *Cancer research* **66**, 2296-2304, doi:10.1158/0008-5472.CAN-05-2216 (2006).

454 Xiang, N., Zhao, R. & Zhong, W. Sodium selenite induces apoptosis by generation of superoxide via the mitochondrial-dependent pathway in human prostate cancer cells. *Cancer chemotherapy and pharmacology* **63**, 351-362, doi:10.1007/s00280-008-0745-3 (2009).

455 Li, G. X., Hu, H., Jiang, C., Schuster, T. & Lu, J. Differential involvement of reactive oxygen species in apoptosis induced by two classes of selenium compounds in human prostate cancer cells. *Int J Cancer* **120**, 2034-2043, doi:10.1002/ijc.22480 (2007).

456 Wu, Y., Zu, K., Warren, M. A., Wallace, P. K. & Ip, C. Delineating the mechanism by which selenium deactivates Akt in prostate cancer cells. *Molecular cancer therapeutics* **5**, 246-252, doi:10.1158/1535-7163.MCT-05-0376 (2006).

457 Zhao, H. & Brooks, J. D. Selenomethionine induced transcriptional programs in human prostate cancer cells. *The Journal of urology* **177**, 743-750, doi:10.1016/j.juro.2006.09.071 (2007).

458 Xiang, N., Zhao, R., Song, G. & Zhong, W. Selenite reactivates silenced genes by modifying DNA methylation and histones in prostate cancer cells. *Carcinogenesis* **29**, 2175-2181, doi:10.1093/carcin/bgn179 (2008).

459 Berggren, M. *et al.* Sodium selenite increases the activity of the tumor suppressor protein, PTEN, in DU-145 prostate cancer cells. *Nutrition and cancer* **61**, 322-331, doi:10.1080/01635580802521338 (2009).
460 Hurst, R., Elliott, R. M., Goldson, A. J. & Fairweather-Tait, S. J. Se-methylselenocysteine alters collagen gene and protein expression in human prostate cells. *Cancer letters* **269**, 117-126, doi:10.1016/j.canlet.2008.04.025 (2008).
461 Chun, J. Y. *et al.* Selenium inhibition of survivin expression by preventing Sp1 binding to its promoter. *Molecular cancer therapeutics* **6**, 2572-2580, doi:10.1158/1535-7163.MCT-07-0172 (2007).
462 Liu, X. *et al.* Survivin gene silencing sensitizes prostate cancer cells to selenium growth inhibition. *BMC cancer* **10**, 418, doi:10.1186/1471-2407-10-418 (2010).
463 Pei, Z., Li, H., Guo, Y., Jin, Y. & Lin, D. Sodium selenite inhibits the expression of VEGF, TGFbeta(1) and IL-6 induced by LPS in human PC3 cells via TLR4-NF-(K)B signaling blockage. *International immunopharmacology* **10**, 50-56, doi:10.1016/j.intimp.2009.09.020 (2010).
464 Sinha, I. *et al.* Methylseleninic acid downregulates hypoxia-inducible factor-1a in invasive prostate cancer. *Int J Cancer* **130**, 1430-1439, doi:10.1002/ijc.26141 (2012).
465 Sinha, I., Allen, J. E., Pinto, J. T. & Sinha, R. Methylseleninic acid elevates REDD1 and inhibits prostate cancer cell growth despite AKT activation and mTOR dysregulation in hypoxia. *Cancer medicine* **3**, 252-264, doi:10.1002/cam4.198 (2014).
466 Corcoran, N. M., Najdovska, M. & Costello, A. J. Inorganic selenium retards progression of experimental hormone refractory prostate cancer. *The Journal of urology* **171**, 907-910, doi:10.1097/01.ju.0000092859.16817.8e (2004).
467 Li, G. X. *et al.* Superior in vivo inhibitory efficacy of methylseleninic acid against human prostate cancer over selenomethionine or selenite. *Carcinogenesis* **29**, 1005-1012, doi:10.1093/carcin/bgn007 (2008).
468 Wang, L. *et al.* Methyl-selenium compounds inhibit prostate carcinogenesis in the transgenic adenocarcinoma of mouse prostate model with survival benefit. *Cancer prevention research* **2**, 484-495, doi:10.1158/1940-6207.CAPR-08-0173 (2009).
469 Lindshield, B. L. *et al.* Selenium, but Not Lycopene or Vitamin E, Decreases Growth of Transplantable Dunning R3327-H Rat Prostate Tumors. *PloS one* **5**, doi:ARTN e10423

10.1371/journal.pone.0010423 (2010).
470 Wang, L. *et al.* Methylseleninic Acid Superactivates p53-Senescence Cancer Progression Barrier in Prostate Lesions of Pten-Knockout Mouse. *Cancer prevention research* **9**, 35-42, doi:10.1158/1940-6207.CAPR-15-0236 (2016).
471 Venkateswaran, V., Fleshner, N. E., Sugar, L. M. & Klotz, L. H. Antioxidants block prostate cancer in lady transgenic mice. *Cancer research* **64**, 5891-5896, doi:10.1158/0008-5472.CAN-04-0690 (2004).
472 Bhattacharyya, R. S., Husbeck, B., Feldman, D. & Knox, S. J. Selenite treatment inhibits LAPC-4 tumor growth and prostate-specific antigen secretion in a xenograft model of human prostate cancer. *International journal of radiation oncology, biology, physics* **72**, 935-940, doi:10.1016/j.ijrobp.2008.07.005 (2008).
473 Lee, S. O. *et al.* Monomethylated selenium inhibits growth of LNCaP human prostate cancer xenograft accompanied by a decrease in the expression of androgen receptor and prostate-specific antigen (PSA). *The Prostate* **66**, 1070-1075, doi:10.1002/pros.20329 (2006).

474 Ozten, N., Horton, L., Lasano, S. & Bosland, M. C. Selenomethionine and alpha-Tocopherol Do Not Inhibit Prostate Carcinogenesis in the Testosterone plus Estradiol-Treated NBL Rat Model. *Cancer prevention research* **3**, 371-380, doi:10.1158/1940-6207.CAPR-09-0152 (2010).

475 Ozten, N., Schlicht, M., Diamond, A. M. & Bosland, M. C. L-Selenomethionine Does Not Protect Against Testosterone Plus 17 beta-Estradiol-Induced Oxidative Stress and Preneoplastic Lesions in the Prostate of NBL Rats. *Nutrition and Cancer-an International Journal* **66**, 825-834, doi:10.1080/01635581.2014.904907 (2014).

476 McCormick, D. L. *et al.* Null Activity of Selenium and Vitamin E as Cancer Chemopreventive Agents in the Rat Prostate. *Cancer prevention research* **3**, 381-392, doi:10.1158/1940-6207.CAPR-09-0176 (2010).

477 Singh, C. K. *et al.* Methaneseleninic acid and gamma-Tocopherol combination inhibits prostate tumor growth in Vivo in a xenograft mouse model. *Oncotarget* **5**, 3651-3661, doi:DOI 10.18632/oncotarget.1979 (2014).

478 Zu, K. & Ip, C. Synergy between selenium and vitamin E in apoptosis induction is associated with activation of distinctive initiator caspases in human prostate cancer cells. *Cancer research* **63**, 6988-6995 (2003).

479 Venkateswaran, V., Fleshner, N. E. & Klotz, L. H. Synergistic effect of vitamin E and selenium in human prostate cancer cell lines. *Prostate cancer and prostatic diseases* **7**, 54-56, doi:10.1038/sj.pcan.4500707 (2004).

480 Lippman, S. M. *et al.* Designing the Selenium and Vitamin E Cancer Prevention Trial (SELECT). *Journal of the National Cancer Institute* **97**, 94-102, doi:10.1093/jnci/dji009 (2005).

481 Alpha-Tocopherol, B. C. C. P. S. G. The effect of vitamin E and beta carotene on the incidence of lung cancer and other cancers in male smokers. *The New England journal of medicine* **330**, 1029-1035, doi:10.1056/NEJM199404143301501 (1994).

482 Heinonen, O. P. *et al.* Prostate cancer and supplementation with alpha-tocopherol and beta-carotene: incidence and mortality in a controlled trial. *Journal of the National Cancer Institute* **90**, 440-446 (1998).

483 Weinstein, S. J. *et al.* Serum and dietary vitamin E in relation to prostate cancer risk. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology* **16**, 1253-1259, doi:10.1158/1055-9965.EPI-06-1084 (2007).

484 Virtamo, J. *et al.* Incidence of cancer and mortality following alpha-tocopherol and beta-carotene supplementation: a postintervention follow-up. *Jama* **290**, 476-485, doi:10.1001/jama.290.4.476 (2003).

485 Peters, U. *et al.* Vitamin E and selenium supplementation and risk of prostate cancer in the Vitamins and lifestyle (VITAL) study cohort. *Cancer causes & control : CCC* **19**, 75-87, doi:10.1007/s10552-007-9072-y (2008).

486 Chan, J. M. *et al.* Supplemental vitamin E intake and prostate cancer risk in a large cohort of men in the United States. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology* **8**, 893-899 (1999).

487 Rodriguez, C. *et al.* Vitamin E supplements and risk of prostate cancer in U.S. men. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology* **13**, 378-382 (2004).

488 Nicastro, H. L. & Dunn, B. K. Selenium and prostate cancer prevention: insights from the selenium and vitamin E cancer prevention trial (SELECT). *Nutrients* **5**, 1122-1148, doi:10.3390/nu5041122 (2013).
489 Yu, S. Y., Zhu, Y. J. & Li, W. G. Protective role of selenium against hepatitis B virus and primary liver cancer in Qidong. *Biological trace element research* **56**, 117-124 (1997).
490 Blot, W. J. *et al.* Nutrition intervention trials in Linxian, China: supplementation with specific vitamin/mineral combinations, cancer incidence, and disease-specific mortality in the general population. *Journal of the National Cancer Institute* **85**, 1483-1492 (1993).
491 Li, J. Y. *et al.* Nutrition Intervention Trials in Linxian, China - Multiple Vitamin Mineral Supplementation, Cancer Incidence, and Disease-Specific Mortality among Adults with Esophageal Dysplasia. *Journal of the National Cancer Institute* **85**, 1492-1498, doi:DOI 10.1093/jnci/85.18.1492 (1993).
492 Clark, L. C. *et al.* Effects of selenium supplementation for cancer prevention in patients with carcinoma of the skin. A randomized controlled trial. Nutritional Prevention of Cancer Study Group. *Jama* **276**, 1957-1963 (1996).
493 Clark, L. C. *et al.* Decreased incidence of prostate cancer with selenium supplementation: results of a double-blind cancer prevention trial. *British journal of urology* **81**, 730-734 (1998).
494 Duffield-Lillico, A. J. *et al.* Selenium supplementation, baseline plasma selenium status and incidence of prostate cancer: an analysis of the complete treatment period of the Nutritional Prevention of Cancer Trial. *BJU international* **91**, 608-612 (2003).
495 Lippman, S. M. *et al.* Effect of selenium and vitamin E on risk of prostate cancer and other cancers: the Selenium and Vitamin E Cancer Prevention Trial (SELECT). *Jama* **301**, 39-51, doi:10.1001/jama.2008.864 (2009).
496 Klein, E. A. *et al.* Vitamin E and the risk of prostate cancer: the Selenium and Vitamin E Cancer Prevention Trial (SELECT). *Jama* **306**, 1549-1556, doi:10.1001/jama.2011.1437 (2011).
497 Kristal, A. R. *et al.* Baseline Selenium Status and Effects of Selenium and Vitamin E Supplementation on Prostate Cancer Risk. *Jnci-J Natl Cancer I* **106**, doi:ARTN djt456

10.1093/jnci/djt456 (2014).
498 Potter, J. D. The failure of cancer chemoprevention. *Carcinogenesis* **35**, 974-982, doi:10.1093/carcin/bgu063 (2014).
499 Algotar, A. M. *et al.* Phase 3 clinical trial investigating the effect of selenium supplementation in men at high-risk for prostate cancer. *The Prostate* **73**, 328-335, doi:10.1002/pros.22573 (2013).
500 Moyad, M. A. Selenium and vitamin E supplements for prostate cancer: evidence or embellishment? *Urology* **59**, 9-19 (2002).
501 Ledesma, M. C. *et al.* Selenium and vitamin E for prostate cancer: post-SELECT (Selenium and Vitamin E Cancer Prevention Trial) status. *Molecular medicine* **17**, 134-143, doi:10.2119/molmed.2010.00136 (2011).
502 Hoarau-Vechot, J., Rafii, A., Touboul, C. & Pasquier, J. Halfway between 2D and Animal Models: Are 3D Cultures the Ideal Tool to Study Cancer-Microenvironment Interactions? *International journal of molecular sciences* **19**, doi:10.3390/ijms19010181 (2018).
503 Antoni, D., Burckel, H., Josset, E. & Noel, G. Three-dimensional cell culture: a breakthrough in vivo. *International journal of molecular sciences* **16**, 5517-5527, doi:10.3390/ijms16035517 (2015).

504 Mroue, R. & Bissell, M. J. Three-dimensional cultures of mouse mammary epithelial cells. *Methods in molecular biology* **945**, 221-250, doi:10.1007/978-1-62703-125-7_14 (2013).
505 Edmondson, R., Broglie, J. J., Adcock, A. F. & Yang, L. Three-dimensional cell culture systems and their applications in drug discovery and cell-based biosensors. *Assay and drug development technologies* **12**, 207-218, doi:10.1089/adt.2014.573 (2014).
506 Giangreco, A. A. *et al.* Differential expression and regulation of vitamin D hydroxylases and inflammatory genes in prostate stroma and epithelium by 1,25-dihydroxyvitamin D in men with prostate cancer and an in vitro model. *The Journal of steroid biochemistry and molecular biology* **148**, 156-165, doi:10.1016/j.jsbmb.2014.10.004 (2015).
507 Mihelich, B. L. *et al.* miR-183-96-182 Cluster Is Overexpressed in Prostate Tissue and Regulates Zinc Homeostasis in Prostate Cells. *Journal of Biological Chemistry* **286**, 44503-44511, doi:10.1074/jbc.M111.262915 (2011).
508 Peehl, D. M. Are primary cultures realistic models of prostate cancer? *Journal of cellular biochemistry* **91**, 185-195, doi:10.1002/jcb.10691 (2004).
509 Unno, K. *et al.* Modeling African American prostate adenocarcinoma by inducing defined genetic alterations in organoids. *Oncotarget*, doi:10.18632/oncotarget.17230 (2017).
510 Li, H. & Durbin, R. Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics* **25**, 1754-1760, doi:10.1093/bioinformatics/btp324 (2009).
511 Stenson, P. D. *et al.* Human Gene Mutation Database (HGMD): 2003 update. *Human mutation* **21**, 577-581, doi:10.1002/humu.10212 (2003).
512 Landrum, M. J. *et al.* ClinVar: public archive of relationships among sequence variation and human phenotype. *Nucleic acids research* **42**, D980-985, doi:10.1093/nar/gkt1113 (2014).
513 Green, R. C. *et al.* ACMG recommendations for reporting of incidental findings in clinical exome and genome sequencing. *Genetics in medicine : official journal of the American College of Medical Genetics* **15**, 565-574, doi:10.1038/gim.2013.73 (2013).
514 Subramanian, A. *et al.* Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 15545-15550, doi:10.1073/pnas.0506580102 (2005).
515 Merico, D., Isserlin, R., Stueker, O., Emili, A. & Bader, G. D. Enrichment map: a network-based method for gene-set enrichment visualization and interpretation. *PloS one* **5**, e13984, doi:10.1371/journal.pone.0013984 (2010).
516 Shannon, P. *et al.* Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome research* **13**, 2498-2504, doi:10.1101/gr.1239303 (2003).
517 Folkman, J. & Moscona, A. Role of cell shape in growth control. *Nature* **273**, 345-349 (1978).
518 Dyson, N., Howley, P. M., Munger, K. & Harlow, E. The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. *Science* **243**, 934-937 (1989).
519 Bello, D., Webber, M. M., Kleinman, H. K., Wartinger, D. D. & Rhim, J. S. Androgen responsive adult human prostatic epithelial cell lines immortalized by human papillomavirus 18. *Carcinogenesis* **18**, 1215-1223 (1997).
520 Whillier, S., Raftos, J. E., Chapman, B. & Kuchel, P. W. Role of N-acetylcysteine and cystine in glutathione synthesis in human erythrocytes. *Redox report : communications in free radical research* **14**, 115-124, doi:10.1179/135100009X392539 (2009).
521 Girotti, A. W. Lipid hydroperoxide generation, turnover, and effector action in biological systems. *Journal of lipid research* **39**, 1529-1542 (1998).

522 Schafer, Z. T. *et al.* Antioxidant and oncogene rescue of metabolic defects caused by loss of matrix attachment. *Nature* **461**, 109-113, doi:10.1038/nature08268 (2009).

523 Frisch, S. M. & Francis, H. Disruption of epithelial cell-matrix interactions induces apoptosis. *The Journal of cell biology* **124**, 619-626 (1994).

524 Rotem, A. *et al.* Alternative to the soft-agar assay that permits high-throughput drug and genetic screens for cellular transformation. *Proceedings of the National Academy of Sciences of the United States of America* **112**, 5708-5713, doi:10.1073/pnas.1505979112 (2015).

525 Fukazawa, H., Nakano, S., Mizuno, S. & Uehara, Y. Inhibitors of anchorage-independent growth affect the growth of transformed cells on poly(2-hydroxyethyl methacrylate)-coated surfaces. *Int J Cancer* **67**, 876-882, doi:10.1002/(SICI)1097-0215(19960917)67:6<876::AID-IJC19>3.0.CO;2-# (1996).

526 Grassian, A. R., Coloff, J. L. & Brugge, J. S. Extracellular matrix regulation of metabolism and implications for tumorigenesis. *Cold Spring Harbor symposia on quantitative biology* **76**, 313-324, doi:10.1101/sqb.2011.76.010967 (2011).

527 Coloff, J. L. *et al.* Differential Glutamate Metabolism in Proliferating and Quiescent Mammary Epithelial Cells. *Cell metabolism* **23**, 867-880, doi:10.1016/j.cmet.2016.03.016 (2016).

528 Caneba, C. A., Bellance, N., Yang, L. F., Pabst, L. & Nagrath, D. Pyruvate uptake is increased in highly invasive ovarian cancer cells under anoikis conditions for anaplerosis, mitochondrial function, and migration. *Am J Physiol-Endoc M* **303**, E1036-E1052, doi:10.1152/ajpendo.00151.2012 (2012).

529 Shi, Z., Wang, B., Chihanga, T., Kennedy, M. A. & Weber, G. F. Energy metabolism during anchorage-independence. Induction by osteopontin-c. *PloS one* **9**, e105675, doi:10.1371/journal.pone.0105675 (2014).

530 Duval, K. *et al.* Modeling Physiological Events in 2D vs. 3D Cell Culture. *Physiology* **32**, 266-277, doi:10.1152/physiol.00036.2016 (2017).

531 Yamada, K. M. & Cukierman, E. Modeling tissue morphogenesis and cancer in 3D. *Cell* **130**, 601-610, doi:10.1016/j.cell.2007.08.006 (2007).

532 Pickup, M. W., Mouw, J. K. & Weaver, V. M. The extracellular matrix modulates the hallmarks of cancer. *Embo Rep* **15**, 1243-1253, doi:10.15252/embr.201439246 (2014).

533 Xia, J. *et al.* Semaphorin-Plexin Signaling Controls Mitotic Spindle Orientation during Epithelial Morphogenesis and Repair. *Developmental cell* **33**, 299-313, doi:10.1016/j.devcel.2015.02.001 (2015).

534 Toivanen, R. & Shen, M. M. Prostate organogenesis: tissue induction, hormonal regulation and cell type specification. *Development* **144**, 1382-1398, doi:10.1242/dev.148270 (2017).

535 Lancaster, M. A. & Knoblich, J. A. Organogenesis in a dish: modeling development and disease using organoid technologies. *Science* **345**, 1247125, doi:10.1126/science.1247125 (2014).

536 Bissell, M. J., Rizki, A. & Mian, I. S. Tissue architecture: the ultimate regulator of breast epithelial function. *Current opinion in cell biology* **15**, 753-762 (2003).

537 Webber, M. M., Bello, D., Kleinman, H. K. & Hoffman, M. P. Acinar differentiation by non-malignant immortalized human prostatic epithelial cells and its loss by malignant cells. *Carcinogenesis* **18**, 1225-1231 (1997).

538 Zhang, X. *et al.* Inhibition of vimentin or beta1 integrin reverts morphology of prostate tumor cells grown in laminin-rich extracellular matrix gels and reduces tumor growth in vivo. *Molecular cancer therapeutics* **8**, 499-508, doi:10.1158/1535-7163.MCT-08-0544 (2009).

539 Li, C. R. *et al.* Molecular Profiling of Prostatic Acinar Morphogenesis Identifies PDCD4 and KLF6 as Tissue Architecture-Specific Prognostic Markers in Prostate Cancer. *American Journal of Pathology* **182**, 363-374, doi:10.1016/j.ajpath.2012.10.024 (2013).
540 Bandy, B. & Davison, A. J. Mitochondrial mutations may increase oxidative stress: implications for carcinogenesis and aging? *Free radical biology & medicine* **8**, 523-539 (1990).
541 Martinez, E. E., Anderson, P. D., Logan, M. & Abdulkadir, S. A. Antioxidant treatment promotes prostate epithelial proliferation in Nkx3.1 mutant mice. *PloS one* **7**, e46792, doi:10.1371/journal.pone.0046792 (2012).
542 Martinez, E. E. *et al.* A functional variant in NKX3.1 associated with prostate cancer risk in the Selenium and Vitamin E Cancer Prevention Trial (SELECT). *Cancer prevention research* **7**, 950-957, doi:10.1158/1940-6207.CAPR-14-0075 (2014).
543 Njoroge, R. N. *et al.* Organoids model distinct Vitamin E effects at different stages of prostate cancer evolution. *Sci Rep-Uk* **7**, doi:Artn 16285

10.1038/S41598-017-16459-2 (2017).
544 Achilli, T. M., Meyer, J. & Morgan, J. R. Advances in the formation, use and understanding of multi-cellular spheroids. *Expert opinion on biological therapy* **12**, 1347-1360, doi:10.1517/14712598.2012.707181 (2012).
545 Fung, C., Lock, R., Gao, S., Salas, E. & Debnath, J. Induction of autophagy during extracellular matrix detachment promotes cell survival. *Molecular biology of the cell* **19**, 797-806, doi:10.1091/mbc.E07-10-1092 (2008).
546 Chen, N. & Debnath, J. IkappaB kinase complex (IKK) triggers detachment-induced autophagy in mammary epithelial cells independently of the PI3K-AKT-MTORC1 pathway. *Autophagy* **9**, 1214-1227, doi:10.4161/auto.24870 (2013).
547 Geiger, B., Bershadsky, A., Pankov, R. & Yamada, K. M. Transmembrane crosstalk between the extracellular matrix--cytoskeleton crosstalk. *Nature reviews. Molecular cell biology* **2**, 793-805, doi:10.1038/35099066 (2001).
548 Xia, H., Nho, R. S., Kahm, J., Kleidon, J. & Henke, C. A. Focal adhesion kinase is upstream of phosphatidylinositol 3-kinase/Akt in regulating fibroblast survival in response to contraction of type I collagen matrices via a beta(1) integrin viability signaling pathway. *Journal of Biological Chemistry* **279**, 33024-33034, doi:10.1074/jbc.M313265200 (2004).
549 Mitra, S. K., Hanson, D. A. & Schlaepfer, D. D. Focal adhesion kinase: in command and control of cell motility. *Nature reviews. Molecular cell biology* **6**, 56-68, doi:10.1038/nrm1549 (2005).
550 Cabodi, S. *et al.* Integrin regulation of epidermal growth factor (EGF) receptor and of EGF-dependent responses. *Biochemical Society transactions* **32**, 438-442, doi:10.1042/BST0320438 (2004).
551 Paoli, P., Giannoni, E. & Chiarugi, P. Anoikis molecular pathways and its role in cancer progression. *Biochimica et biophysica acta* **1833**, 3481-3498, doi:10.1016/j.bbamcr.2013.06.026 (2013).
552 Vlahakis, A. & Debnath, J. The Interconnections between Autophagy and Integrin-Mediated Cell Adhesion. *Journal of molecular biology* **429**, 515-530, doi:10.1016/j.jmb.2016.11.027 (2017).
553 Debnath, J. *et al.* The role of apoptosis in creating and maintaining luminal space within normal and oncogene-expressing mammary acini (vol 111, pg 29, 2002). *Cell* **111**, 757-757, doi:Doi 10.1016/S0092-8674(02)01163-7 (2002).

554 Sheikh, F. G., Pahan, K., Khan, M., Barbosa, E. & Singh, I. Abnormality in catalase import into peroxisomes leads to severe neurological disorder. *Proceedings of the National Academy of Sciences of the United States of America* **95**, 2961-2966, doi:DOI 10.1073/pnas.95.6.2961 (1998).

555 Gulati, S., Ainol, L., Orak, J., Singh, A. K. & Singh, I. Alterations of peroxisomal function in ischemia-reperfusion injury of rat kidney. *Biochimica et biophysica acta* **1182**, 291-298 (1993).

556 Hashimoto, F. & Hayashi, H. Significance of catalase in peroxisomal fatty acyl-CoA beta-oxidation: NADH oxidation by acetoacetyl-CoA and H2O2. *Journal of biochemistry* **108**, 426-431 (1990).

557 Xu, X. M. *et al.* Biosynthesis of selenocysteine on its tRNA in eukaryotes. *PLoS biology* **5**, e4, doi:10.1371/journal.pbio.0050004 (2007).

558 Yuan, J. *et al.* RNA-dependent conversion of phosphoserine forms selenocysteine in eukaryotes and archaea. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 18923-18927, doi:10.1073/pnas.0609703104 (2006).

559 Jiang, L. *et al.* Reductive carboxylation supports redox homeostasis during anchorage-independent growth. *Nature* **532**, 255-258, doi:10.1038/nature17393 (2016).

560 Ishikawa, F., Ushida, K., Mori, K. & Shibanuma, M. Loss of anchorage primarily induces non-apoptotic cell death in a human mammary epithelial cell line under atypical focal adhesion kinase signaling. *Cell death & disease* **6**, e1619, doi:10.1038/cddis.2014.583 (2015).

561 Jamaspishvili, T. *et al.* Clinical implications of PTEN loss in prostate cancer. *Nature reviews. Urology* **15**, 222-234, doi:10.1038/nrurol.2018.9 (2018).

562 Scandroglio, F., Tortora, V., Radi, R. & Castro, L. Metabolic control analysis of mitochondrial aconitase: influence over respiration and mitochondrial superoxide and hydrogen peroxide production. *Free radical research* **48**, 684-693, doi:10.3109/10715762.2014.900175 (2014).

563 Armstrong, J. S., Whiteman, M., Yang, H. & Jones, D. P. The redox regulation of intermediary metabolism by a superoxide-aconitase rheostat. *BioEssays : news and reviews in molecular, cellular and developmental biology* **26**, 894-900, doi:10.1002/bies.20071 (2004).