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Role Of The Cholesterol Metabolite 27-Hydroxycholesterol In Breast And Prostate Cancer

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ROLE OF THE CHOLESTEROL METABOLITE 27-HYDROXYCHOLESTEROL IN BREAST AND PROSTATE CANCER

by

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This dissertation, submitted by Shaneabbas Raza in partial fulfillment of the requirements for the Degree of Doctor of Philosophy from the University of North Dakota, has been read by the Faculty Advisory Committee under whom the work has been done and is hereby approved.

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Shaneabbas Raza

07/10/2016

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To my grandfather, the late Mohammed Hussein Raza

My grandmother, the late Sughra Ali Nathoo

My uncle, the late Vivian Almeida

To my kids:

“Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less”

- Marie Curie (1867-1934)

GLOBAL ABSTRACT

The cholesterol metabolites known as oxysterols play an important role in maintaining cell homeostasis. They play vital roles in inflammation, cell growth, cell signaling, immunity, oxidative stress and aging. Following the discovery that the most abundant oxysterol, 27-hydroxycholesterol (27-OHC) is a Selective Estrogen Receptor Modulator (SERM), it has been implicated in hormonal cancers such as breast and uterine cancer. 27-OHC is a ligand of estrogen receptors (ER), a nuclear hormone receptor involved in cell growth and proliferation. The downstream events followed by 27-OHC-induced ER activation in the context of breast cancer is unknown. Also, the role of 27-OHC in prostate cancer is ill-defined given the involvement of ER in prostate cancer (PCa) and benign prostatic hyperplasia (BPH). Our studies delineate for the first time a potential cellular mechanism of action of 27-OHC in the context of ER+ breast cancer, whereby 27-OHC induced ER activation in ER+ MCF7 cells increases cell proliferation via perturbing the p53-MDM2 axis. We demonstrate that 27-OHC, through ER, exacerbated p53 inactivation via MDM2 resulting in an increase in cell proliferation in ER+ breast cancer cells. Next, we address the possibility of 27-OHC exacerbating prostate cancer cell proliferation. In the context of BPH and PCa, we show that 27-OHC not only increased cell proliferation in tumorigenic cell lines, LNCaP and PC3 but also in the non-tumorigenic cells, RWPE-1. We further demonstrate that 27-OHC- induced cell

proliferation in prostate cells is specifically through ERβ. Given the tissue dependent selective ER modulation of 27-OHC, our novel findings suggest that 27-OHC activates ER signaling in the prostate. Altogether, our findings elucidate and establish the novel role and cellular mechanism of action of the oxysterol 27-OHC in the context of breast and prostate cancer. In the interest of discovering new therapeutic avenues for breast and prostate cancers, our work may aid in the development of novel therapies that could mitigate/halt/alleviate the progression of ER+ breast cancers and prostate cancers.

CHAPTER I

INTRODUCTION

Oxysterols: The Arcane Derivatives of Cholesterol

Cholesterol is a critical component of the cell membranes of the cells. It helps to preserve the fluidity of the cell membranes which is vital to maintain cell homeostasis (Róg and Vattulainen 2014). Two thirds of cholesterol in our bodies are synthesized de novo, while the rest is acquired through dietary sources(Dietschy 1984).

Cholesterol is synthesized de novo in the following steps; first, acetyl CoA is converted to 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). Second, HMGCoA is converted to mevalonate by the rate limiting enzyme, 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCoA reductase). Third, mevalonate is converted to an isoprene based compound, isopentenyl pyrophosphate (IPP) which is later transformed into squalene. Lastly, following a series of reactions, squalene is converted to cholesterol which eventually becomes vital precursor of vitamin D, all steroids and bile salts (Singh et al. 2013; Di Ciaula et al. 2014; King 2016) (Fig.1).

Cholesterol regulation is important to cell survival. Cholesterol can be regulated by the rate limiting HMGCoA reductase enzyme, regulation of the

amount of intracellular free cholesterol by sterol O-acyl transferases (SOAT1 and SOAT2) and regulation of plasma cholesterol levels via Low Density Lipoprotein (LDL) receptor facilitated uptake and High Density Lipoprotein(HDL) reverse transport (Goedeke and Fernández-Hernando 2012; van der Wulp et al. 2013; King 2016). Close observation and regulation of the plasma cholesterol (including HDL and LDL fractions) is the most common physiological indicator that clinicians use to diagnose and curb hypercholesterolemia in patients (Grundy and Bilheimer 1989; Guven et al. 2006; Sonmez et al. 2015).

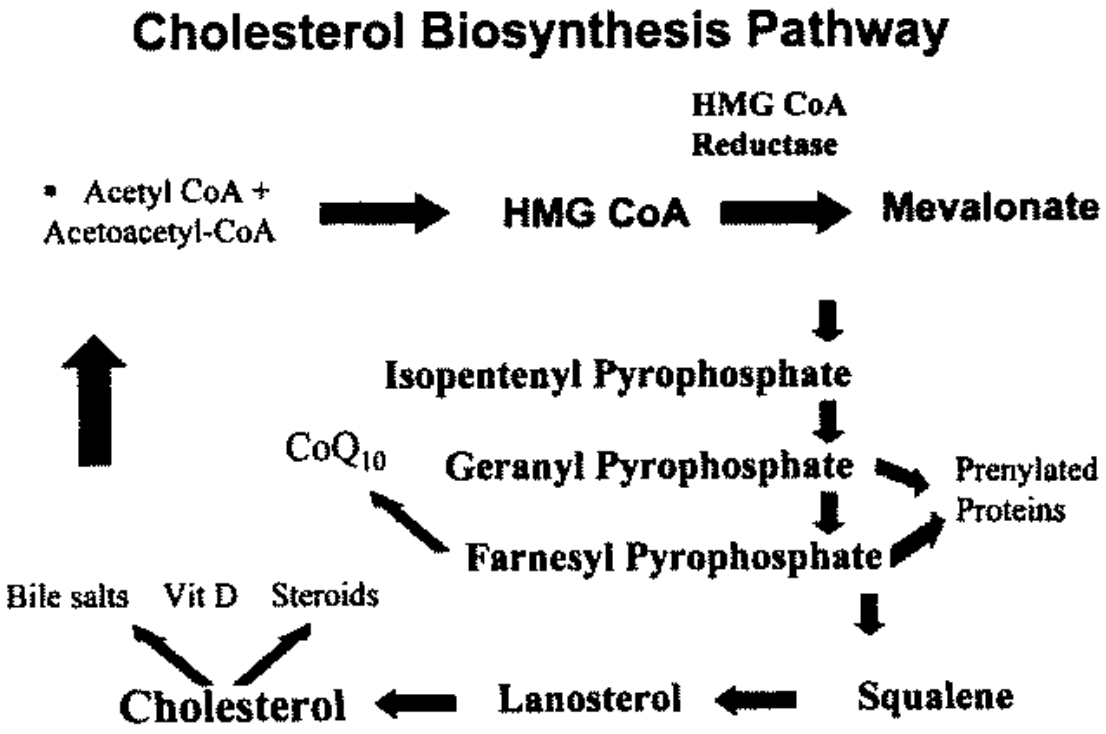


Fig. 1. Cholesterol biosynthesis.

Figure 1: A diagram of the cholesterol biosynthetic pathway. Acetyl CoA converted to HMG CoA which, through a series of reactions leads to the synthesis of cholesterol which is a precursor to bile salts, vitamin D and steroids (reproduced from King 2016).

In addition to being a precursor to steroid hormones (Gabitova et al. 2013), bile acid and vitamin D (Berg et al. 2002), cholesterol is a precursor (as cholesterol continues to exist even after oxidations to oxysterols) to a family of steroid-like compounds called oxysterols. Oxysterols are oxidized cholesterol metabolites which have various signaling consequences in inflammation, immunity, cellular signaling, lipid metabolism, development and apoptosis (Brown and Jessup 1999; Björkhem 2002; Javitt 2008; Sato 2010; Vesa M Olkkonen et al. 2012; Björkhem 2013).

Oxysterols are a 27-carbon compound with a cholesterol like backbone containing either an epoxide or ketone or an additional hydroxyl group in the sterol center and/or a hydroxyl group in the side chain (Vurusaner et al. 2016 Mar) (Fig 2).

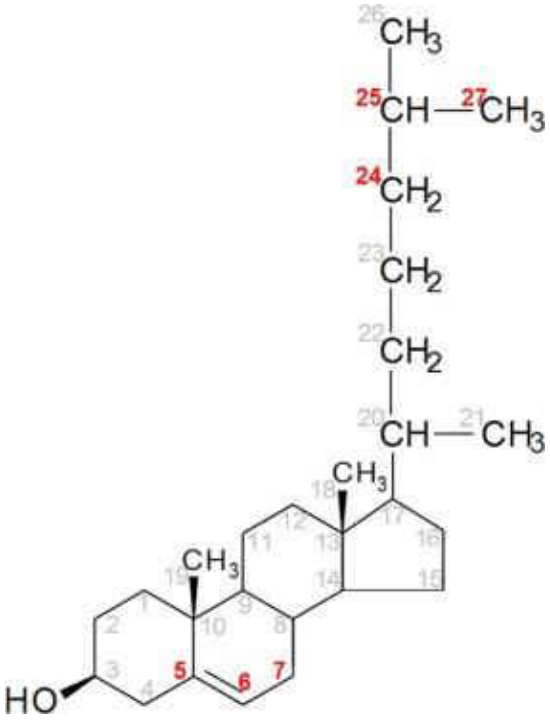


Figure 2: Structure of cholesterol and its main oxidation sites in red. (reproduced from Vurusaner et al. 2016 Mar)

Oxysterols are synthesized by autoxidation or by a specific monooxygenase secondary to enzymatic/non-enzymatic lipid peroxidation. There are several oxysterols generated via cholesterols including but not limited to: 7-hydroepoxycholesterol, 7α-hydroxycholesterol, 7β-hydroxycholesterol, 7-oxocholesterol, cholesterol 5α,6α-epoxide, cholesterol-5β,6β-epoxide, 4β-hydroxycholesterol, cholestane-3β,5α,6β-triol, 27-hydroxycholesterol, 25-hydroxycholesterol, 24S-hydroxycholesterol, 24,25-epoxycholesterol and so forth (Björkhem and Diczfalusy 2002).

The most abundant oxysterol in the brain is 24S-hydroxycholesterol (24S-OHC) while 27 hydroxycholesterol (27-OHC) is predominantly found in the

periphery (Burkard et al. 2007a; Ramirez et al. 2008; Rantham Prabhakara et al. 2008; Hirayama et al. 2009; Björkhem et al. 2013; Bandaru and Haughey 2014). These two oxysterol have been implicated in numerous diseases, particularly in neurodegenerative diseases such as Alzheimer's and Parkinson's disease (Rantham Prabhakara et al. 2008; Dasari et al. 2010; Marwarha et al. 2011; Bandaru and Haughey 2014; Marwarha and Ghribi 2015).

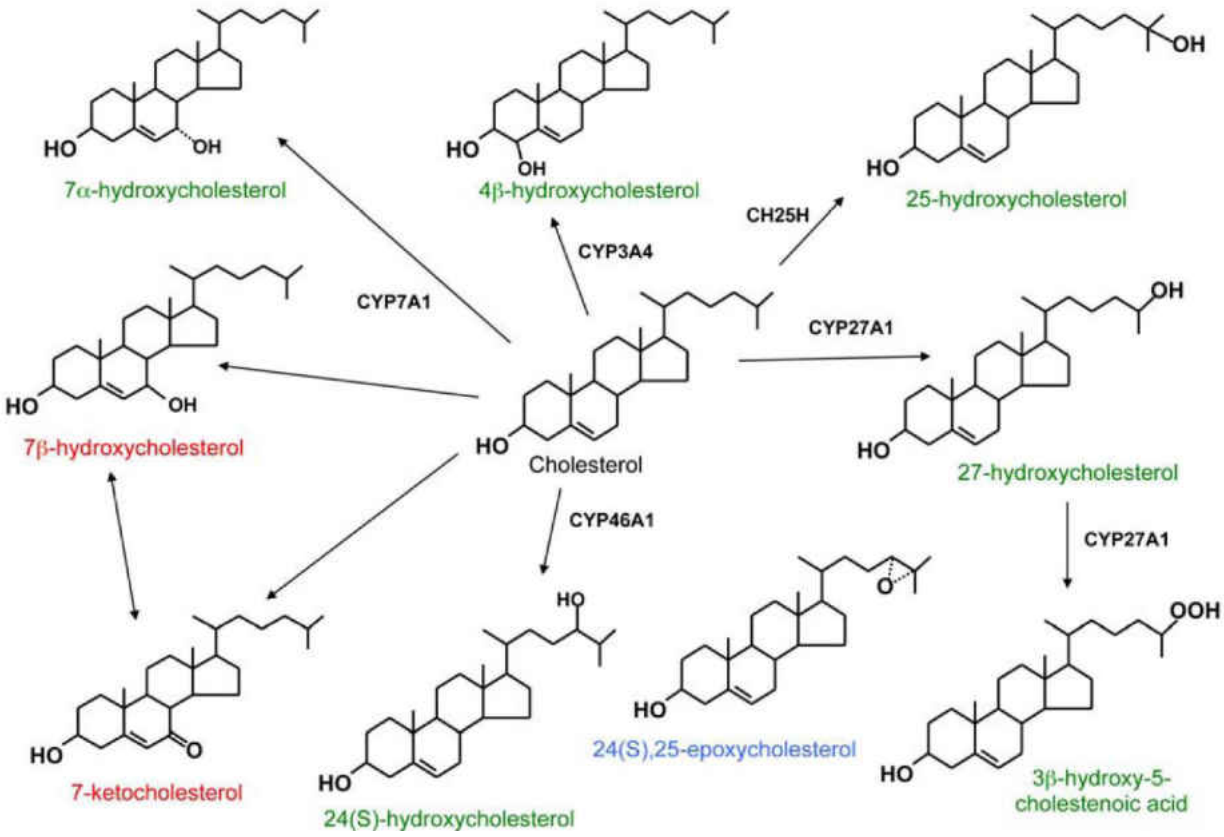


Figure 3: A schematic diagram depicting the biosynthesis of the major oxysterols. The enzymatically derived products are indicated in green, cholesterol metabolites derived from autoxidation with red, and yields derived from shunt of the cholesterol biosynthetic process in blue (reproduced from Olkkonen et al., 2012).

In this dissertation, I will focus on the role of the most abundant oxysterol in the plasma, 27-OHC, in breast and prostate cancers. 27-OHC has been implicated to play significant role in inflammation (Umetani et al. 2014), Alzheimer's disease (Marwarha and Ghribi 2015), Parkinson's disease (Björkhem et al. 2013) and atherosclerosis (Umetani et al. 2007a; Karuna et al. 2011). In this disquisition, I will introduce my findings to establish the newly characterized deleterious role of 27-OHC in hormone-dependent cancers of the breast and prostate.

Breast Cancer

Breast cancer is the most common cancer amongst American women, with one in every eight women developing this disease (Gonzalez-Angulo et al. 2007). The etiology of breast cancer appear to be multi-factorial, including environmental agents and genetic susceptibilities playing a role in the pathophysiology of this disease.

ER alpha and its hormonal ligand, estradiol play a significant role in the development and progression of ER-positive breast cancers (Doisneau-Sixou et al. 2003; Arpino et al. 2005; Bailey et al. 2012; Coates et al. 2012; Renoir et al. 2012) which account for 70% of all breast cancers (Harrell et al. 2006). There are multiple risk factors associated with breast cancer, including hypercholesterolemia (Printz 2014), obesity (James et al. 2015), Type 2 diabetes (Crujeiras et al. 2013) and hormone dyshomeostases (Jankowitz and Davidson 2013; Bodicoat et al. 2014).

Breast cancer develops when the normal breast epithelium transforms into atypical breast hyperplasia, ductal carcinoma *in situ*, invasive ductal carcinoma and eventually a metastatic cancer (Fig. 4). Molecular events leading to the progression at each step of this debilitating disease are yet to be determined, however it is hypothesized that steroidal hormones and their receptors play a vital role in this disease (Rivenbark et al. 2013).

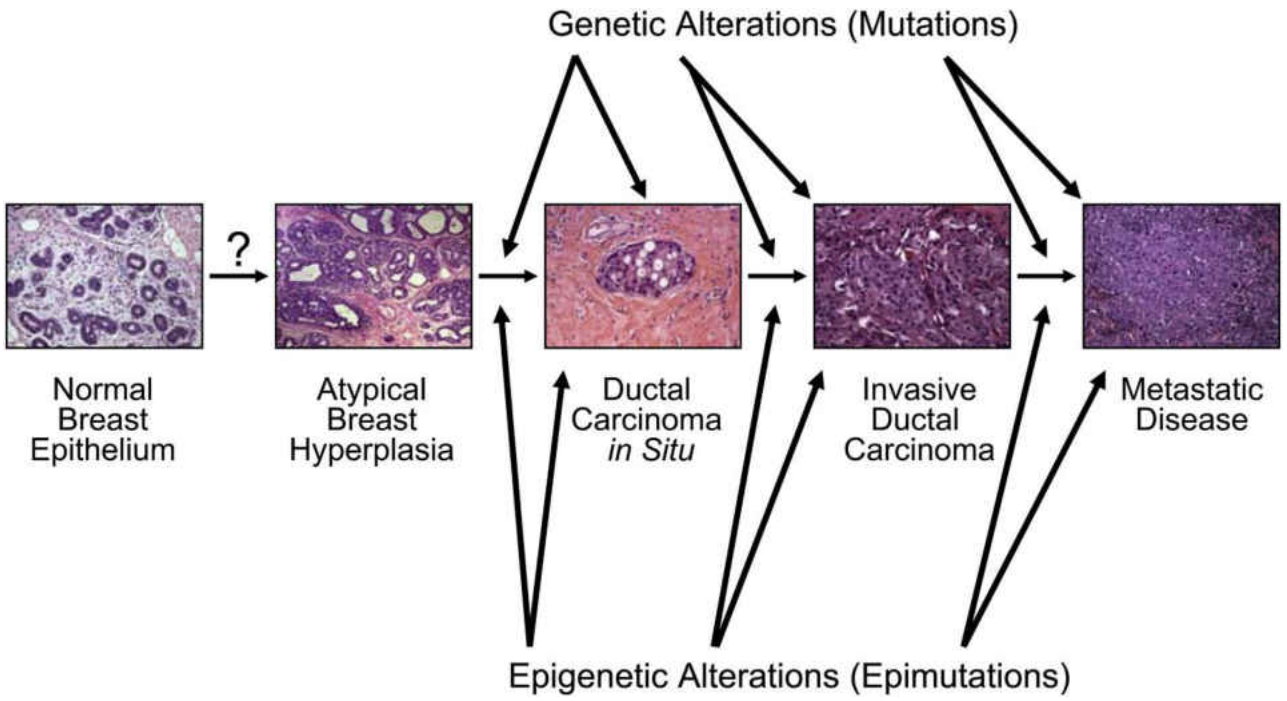


Figure 4: A schematic diagram displaying the canonical progression of breast cancer pathology (reproduced from Gómez-Suaga et al. 2014)

Breast cancers are classified based on patterns of gene expression in the corresponding subtypes. Molecular subtypes have been developed primarily based on five major biomarkers found in the breast tissue: ER, progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2), cytokeratin 5/6 and human epidermal growth factor receptor 1 (HER1). ER+ breast cancers are

sub-classified as luminal A or Luminal B. Luminal A subtype expresses $ER^+/PR^+/HER2^-$, while luminal B expresses $ER^+/PR^+/HER2^+$. Likewise, non-luminal or ER- breast cancers, are subclassified as either $HER2^+$ i.e $ER^-/PR^-/HER2^+$ or $HER2^-$, $ER^-/PR^-/HER2^-$, which is also known as triple negative breast cancer (TNBC) (Fig. 5). Additionally, basal like breast cancers can be identified using the remaining markers: cytokeratin 5/6 and HER1. This method of molecular classification of breast cancer subtypes does not encompass all clinically diagnosed breast cancer cases, however it comprises of most of the breast cancers diagnosed and potentially determines the hormone dependence (or lack thereof) of the breast cancer tumor based on its receptor status (Rivenbark et al. 2013). Other types of breast cancers include HER2 enriched and Claudin-low breast cancers (Prat et al. 2011; Cancer Genome Atlas Network 2012). Patients with ER+ breast cancers are usually treated with anti-estrogens such as tamoxifen, while those with ER- breast cancers are treated with alternative approaches based on HER2 receptor status; for instance, trastuzumab (anti-HER2 drug) to target $HER2^+$ non-luminal breast cancers (Ali and Coombes 2000; Sørlie et al. 2001; Buzdar 2004; Rivenbark et al. 2013).

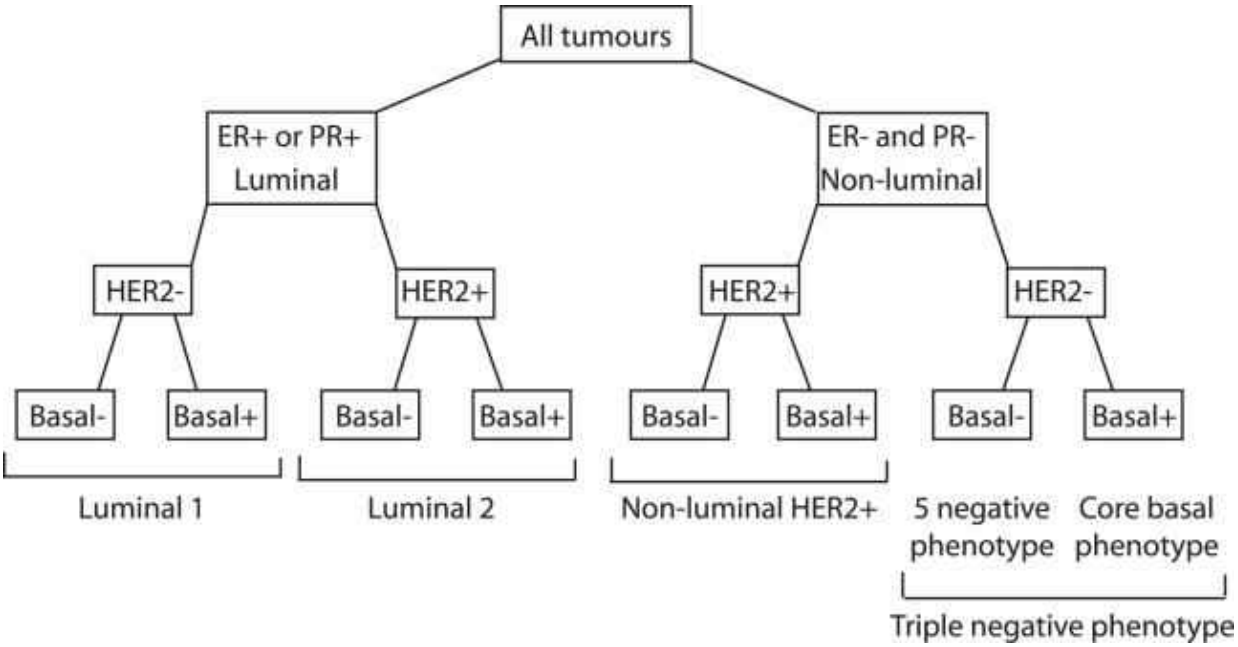


Figure 5: A diagram depicting the classification of all breast cancers based on available immunohistochemistry marker profiles (reproduced from Blows et al. 2010).

ER signaling pathways can be affected by the abundance of estrogens and estrogen like molecules in the body, diet and/or the environment. Uncontrolled ER activation can also occur due to mutations and epigenetic events leading to the development of atypical breast hyperplasia subsequently ER+ breast cancer (Kun et al. 2003; Renoir et al. 2012; Rivenbark et al. 2013).

Currently, for ER+ breast cancers, hormone therapy is the frontline therapeutic avenue utilized by clinicians to combat ER+ breast cancer tumor growth (Baum et al. 2002a; McKeage et al. 2004; Renoir et al. 2012). There are 2 distinct ways by which hormone therapy works: by directly blocking estrogens and estrogen like molecules using competitive ER antagonists and by reducing

the bioavailability of estrogens through inhibiting their biosynthesis via aromatase inhibitors. While both strategies are effective, there is a concern of ER+ breast cancer cells being 'desensitized' and becoming less dependent on estrogens for tumor growth. It is well known that patients often develop resistance to hormone therapy during the course of their treatment and require a combination of alternative therapies (including radiotherapy) to effectively curb ER+ tumor growth and/or metastasis (Hasson et al. 2013; Hart et al. 2015).

The cholesterol metabolite 27-OHC, binds to estrogen receptor (ER) and elicits ER signaling. 27-OHC is characterized as a Selective Estrogen Receptor Modulator (SERM) and provokes ER activation in the breast, several lines of evidence have suggested its deleterious role in ER+ breast cancer progression (Cruz, Torres, María Eugenia Ramírez, et al. 2010; Umetani and Shaul 2011; Nelson et al. 2013; Wu et al. 2013; Raza et al. 2015). 27-OHC not only increased the proliferation of breast cancer cells *in vitro* (Cruz, Torres, María Eugenia Ramírez, et al. 2010; Nelson et al. 2013; Singh et al. 2013; Wu et al. 2013; Raza et al. 2015), but also increased tumor growth in CYP7B1$^{-/-}$/MMTV-PyMT mice, with high levels of 27-OHC. The increased tumor growth was rescued in these mice by the administration of an ER- specific inhibitor, fulvestrant(Nelson et al. 2013). Also, 27-OHC content in ER+ breast cancer tissue is higher when compared to normal breast tissue from breast cancer patients (Wu et al. 2013). These studies underline the importance of 27-OHC induced ER activation in ER+ breast cancer tumor growth.

In this document, we will discuss the role of 27-OHC in ER+ breast cancer cell proliferation and delineate a potential cellular mechanism of action of 27-OHC and its impact on the p53-MDM2 axis in the context of ER+ breast cancers.

Prostate Cancer

Every year almost 230,000 men are diagnosed with prostate cancer. Prostate cancer (PCa) and benign prostatic hyperplasia (BPH) predominantly occur in older men (Chodak 2006; Dhingra and Bhagwat 2011). The etiology of PCa and BPH appear to be multifactorial with age as the most commonly identified major risk factor (Gann 2002; Chokkalingam et al. 2003; William G. Nelson, M.D., Ph.D., Angelo M. De Marzo, M.D., Ph.D., and William B. Isaacs 2003; Bostwick et al. 2004). Other risk factors that contribute to PCa and BPH include oxidative stress (Khandrika et al. 2009), hypercholesterolemia (Moon et al. 2015) and obesity (Burton et al. 2010).

PCa which affects one in every six men in the US, is an ailment that leads to the enlargement of the prostate due to the presence of cancer cells that could metastasize to the bone or the lymph nodes (Sato et al. 1997). On the other hand, BPH is the enlargement of the prostate due to hyperplasia of the prostatic stroma and/or glandular elements. In PCa, the prostate enlargement is not only due to the hyperplasia of stromal cells but also the prostatic epithelial cells. However, PCa and BPH, both have symptoms that overlap each other making it difficult during diagnosis, especially during early stages of PCa (Chokkalingam et al. 2003; Shukla et al. 2016).

PCa and BPH share risk factors associated with them (Fig. 6). However, the relationship between the two is yet to be elucidated. There are two schools of thought, with one claiming PCa and BPH as two separate disorders while the other believes that BPH is a condition that precedes and eventually leads to PCa development (Ørsted and Bojesen 2013). According to the latest and only systematic review to date, which examined 16 case-control studies and 10 cohort studies, patients with BPH have ~2.9 fold increased incidence of PCa (Dai et al. 2016). However, there is currently little to no evidence to suggest that BPH always progresses to PCa. While both conditions have shared risk factors, some risk factors may favor the transformation of BPH to PCa. For instance, inflammation is a risk factor for BPH and PCa, however some studies have established that BPH tissues with inflammatory infiltrates were more likely to advance to PCa than those without inflammation (Higgins and Thompson 2002; Guyatt et al. 2008).

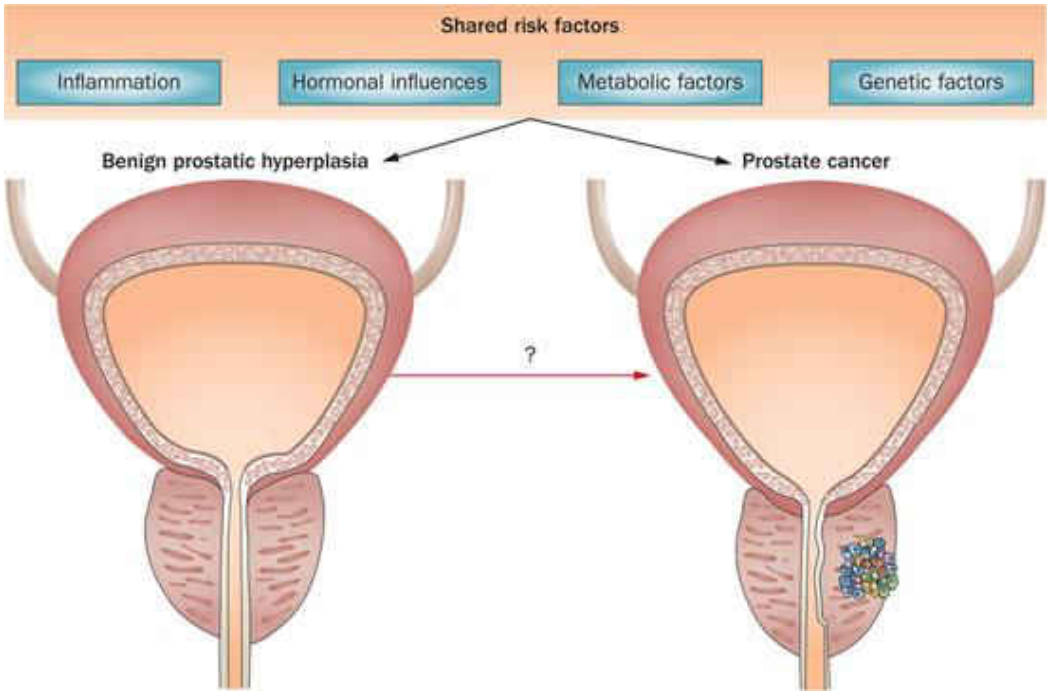


Figure 6: A pictorial diagram depicting the difference and shared risks between BPH and PCa (reproduced from Ørsted and Bojesen 2013).

Urologists currently distinguish between BPH and PCa using a biomarker such as prostate specific antigen (PSA) and histological examination of prostatic tissue. PSA levels are much higher in PCa patients versus BPH patients and presence of pre-cancerous lesions in prostatic biopsies indicate PCa while the absence of it indicates BPH (Geybels et al. 2013; Shukla et al. 2016) (Fig. 7). More non-invasive biomarkers are being identified to better distinguish between PCa and BPH: novel biomarkers include β-2 microglobulin(β2M), pepsinogen 3, group 1 (PGA3) and mucin 3 (MUC3) in the urine (Jedinak et al. 2015).

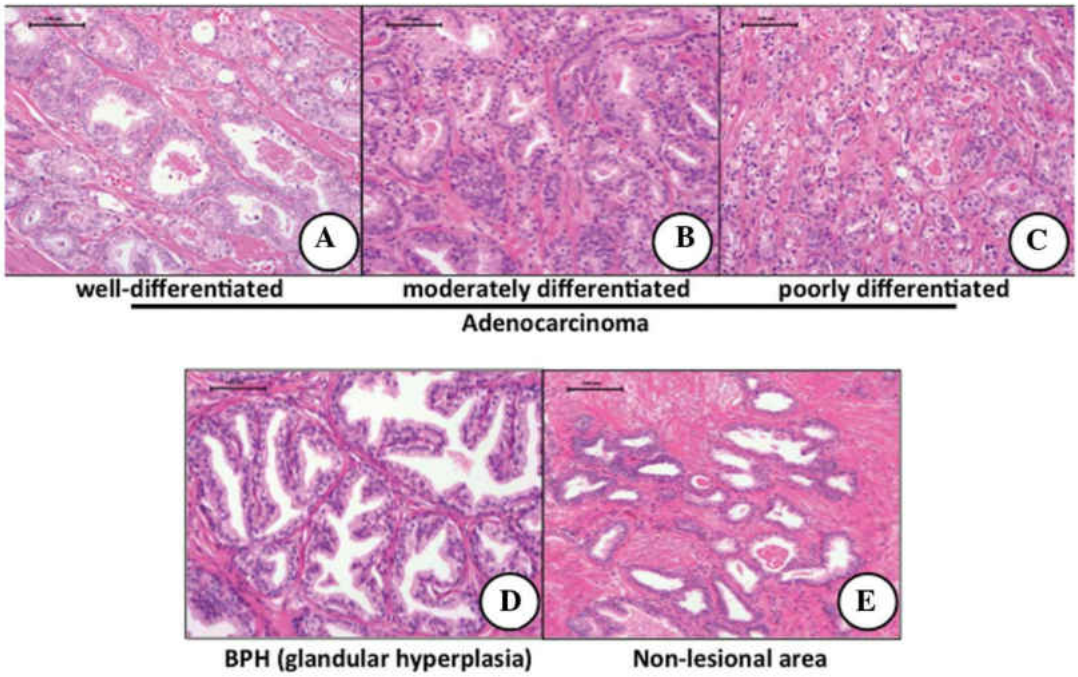


Figure 7: Histopathology of biopsy specimen. Prostate biopsy specimen: (A–C) Adenocarcinoma (magnification, ×100), (D) benign prostate (glandular) hyperplasia and (E) non-lesion area. (hematoxylin and eosin stain; scale bars, 100 μm) (reproduced from Miyazawa et al. 2014)

PCa is an ailment that morphs as it goes through 4 stages of development. The first 2 stages (T1 and T2) include a confined tumor in the prostate. At stage 3 (T3), the tumor invades the seminal vesicles and at stage 4 (T4) the tumor metastasizes to adjacent structures such as the lymph nodes and the bones (Kaarbø et al. 2007) (Fig. 8).

The Stages of Prostate Cancer

STAGE	SUBSTAGE		TYPICAL TREATMENTS
Stage T1 Microscopic tumor confined to prostate gland; palpated gland feels normal	T1a Tumor found in prostate tissue removed for reasons other than cancer; less than 5 percent of specimen is malignant		Observation, surgery to remove the prostate gland (prostatectomy) or local radiation
	T1b Same as T1 but more than 5 percent of specimen contains cancer		Surgery or radiation, possibly combined with hormonal therapy (to stop male hormones from stimulating tumor growth)
	T1c Tumor found through biopsy done in response to an elevated PSA test or to an abnormal ultrasound exam; may be less extensive than a T1b tumor		Same as for T1b
Stage T2 Palpable tumor confined to prostate gland	T2a Tumor confined to less than half of one lobe		Same as for T1b
	T2b Tumor affecting more than half of one lobe		
	T2c Tumor involving both lobes		
Stage T3 Tumor that has begun to expand beyond the prostate	T3a Tumor that protrudes beyond the prostate		Radiation with hormonal therapy; surgery for some patients
	T3b Tumor that has invaded the seminal vesicles		Radiation with hormonal therapy
Stage T4 Tumor that is fixed and has pushed well beyond the prostate into adjacent structures			Hormonal therapy, possibly with radiation to ease local obstructive symptoms; treatment usually is palliative (aimed at slowing disease progression and easing discomfort) rather than curative

Figure 8: Stages of PCa and possible treatment regimens at various stages of the disease (reproduced from Garnick and Fair 1998)

Cholesterol involvement in prostate cancer has been suspected for over a century when it was found to accumulate in prostate cancer tissues (White 1909). More recent studies also demonstrate a positive correlation between high plasma cholesterol and high-grade prostate cancers (Mondul et al. 2010). Diets rich in cholesterol have also been shown to induce metastasis in prostate cancer(Moon et al. 2015). However, statins, a class of drugs which competitively inhibit 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCoA reductase), a key enzyme in cholesterol synthesis pathway, is unable to rescue or predict prognosis of patients with PCa (Geybels et al. 2013; Algotar et al. 2014; Kantor et al. 2015).

The impact of cholesterol in prostate cancer may be through its oxidized metabolite, 27-OHC. Levels of 27-OHC increase with age, oxidative stress, and hypercholesterolemia (Hirayama et al. 2009; Dasari et al. 2010; Prasanthi et al. 2011; Nelson et al. 2013). These factors are all suspected to increase the risk of developing PCa and BPH. In this thesis**,** I will also discuss the potential role of 27-OHC in prostate cancer cell proliferation.

CHAPTER II

ROLE OF 27-HYDROXYCHOLESTEROL IN BREAST CANCER

27-hydroxycholesterol Regulates p53 activity and Increases Cell Proliferation via MDM2 in Breast Cancer Cells.

Abstract

Estrogen is synthesized from cholesterol and high cholesterol levels are suggested to be associated with increased risk of estrogen receptor(ER)-positive breast cancer. The cholesterol metabolite 27-hydroxycholesterol (27-OHC) was recently identified as a selective estrogen receptor modulator (SERM) and has been shown to impact breast cancer progression. However, the mechanisms by which 27-OHC may contribute to breast cancer are not all known. We determined the extent to which 27-OHC regulates cell proliferation in MCF7 ER positive breast cancer cell line involving the tumor suppressor protein p53. We found that treatment of MCF7 cells with 27-OHC reduced p53 transcriptional activity. Conversely, treatment of the ER negative MDA MB 231 cells with 27-OHC induced no change in p53 activity. Exposure of MCF7 cells to 27-OHC was associated with increased protein levels of the E3 ubiquitin protein ligase MDM2 and decreased levels of p53. Moreover, 27-OHC also enhanced physical interaction between p53 and MDM2. Furthermore, 27-OHC induced proliferation was attenuated using either the p53 activator Tenovin-1 or

the MDM2 inhibitor Nutlin-3 and Mdm2 siRNA. Taken together, our results indicate that 27-OHC may contribute to ER positive breast cancer progression by disrupting constitutive p53 signaling in an MDM2 dependent manner.

Introduction

Breast cancer is the most common cancer amongst American women, with one in every eight women developing this disease (Gonzalez-Angulo et al. 2007). The causes of breast cancer are multi-factorial, including environmental agents and genetic susceptibilities. ER alpha and its agonists, estradiol (and estradiol-like compounds), play a significant role in the progression of ER-positive breast cancer forms. Several therapeutic strategies have been developed against ER, however a significant number of ER-positive breast carcinoma patients experience drug resistance (Baum et al. 2002b; Buzdar 2004; Franco et al. 2004).

27-OHC has been characterized as an endogenous ligand for ER (Umetani and Shaul 2011; Nelson et al. 2013; Wu et al. 2013). 27-OHC is an oxysterol formed from cholesterol through the enzyme CYP27A1. This oxysterol is the most abundant cholesterol metabolite in plasma, and also accumulates in macrophages. *In vitro*, 27-OHC elicits a signaling response via ER at concentrations as low as 0.1μM (Wu et al. 2013). In the plasma of healthy human subjects, 27-OHC is found at concentrations of 0.2-0.6μM, and these concentrations can increase dramatically under conditions such as hypercholesterolemia (Duane and Javitt 1999). Thus, fluctuations in 27-OHC may modulate ER and potentially contribute to the pathogenesis of ER+ breast

cancers. While it has been established that 27-OHC is an endogenous selective ER modulator (SERM) and that it exacerbates breast cancer pathophysiology (DuSell et al. 2008; Cruz, Torres, María Eugenia Ramírez, et al. 2010; Nelson et al. 2013), its role in molecular events following ER activation in the context of breast cancer pathogenesis is not fully understood.

The tumor suppressor protein, p53 plays an important role in apoptosis, cell cycle and senescence. Under normal conditions, wild type p53 is in “stand by” mode. Under genotoxic stress, p53 is activated to prevent anomalous cell proliferation and neoplastic development. Hence, p53 has been extensively studied as an anticancer target and as a cancer prognostic tool to diagnose and treat several types of cancers (Pharoah et al. 1999; van der Burg et al. 2001; Miller et al. 2005; Coates et al. 2012; Walerych et al. 2012; Ahn et al. 2013; Khoo et al. 2014). Levels of p53 are regulated by the E3 ubiquitin ligase, Mouse Double Minute 2 protein (MDM2). MDM2 tags p53 to undergo ubiquitination and subsequently proteasomal degradation (Moll and Petrenko 2003; Manfredi 2010; Dolfi et al. 2014). In this report, we determined the effects of 27-OHC on cell proliferation in the context of p53 and MDM2 regulation. We found that 27-OHC via ER inhibits p53 transcriptional activity in an MDM2 dependent manner, resulting in cell proliferation.

Methods

Reagents

27-OHC was purchased from Santa Cruz Biotechnologies (Santa Cruz, CA), the p53 activator, tenovin-1 from Tocris Bioscience (Ellisville, MO), Nutlin-3 and Fulvestrant from Cayman Chemicals (Ann Arbor, MI), the reporter constructs encoding p53 response elements conjugated to the firefly luciferase gene from SA Biosciences (Frederick, MD) and β-estradiol from Sigma-Aldrich (St. Louis, MO). All cell culture reagents, with the exception of fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) were from Invitrogen (Carlsbad, CA). Human MCF7 and MDA MB 231 cell lines were purchased from ATCC (Manassas, VA).

Cell Culture

The human ER-positive breast cancer cells MCF7 and ER-negative breast cancer cells MDA MB 231 were grown in phenol red free DMEM/F12 medium containing 10% charcoal dextran stripped fetal bovine serum (FBS) and 1% antibiotic/antimycotic mix. Cells were maintained at 37°C in a saturated humidity atmosphere containing 95% air and 5% CO_2.

Cell Proliferation Assays

Proliferation assays were conducted on black 96 well plates using CyQUANT Direct Cell Proliferation Assay purchased from Invitrogen (Carlsbad, CA), which quantifies cell number using DNA content and membrane integrity. Cells were processed for proliferation as per manufacturer's protocol and read using Spectra MAX GEMINI EM (Molecular Devices).

Dual Luciferase Assays

Dual Luciferase Reporter Assay System (Promega; Madison, WI) was used to determine the effect of 27-OHC on p53 activity. MCF7 and MDA-MB-231cells were incubated for 18 hours with transfection ready p53 response element conjugated with firefly luciferase construct and constitutively expressing Renilla Luciferase construct (SA biosciences; Valencia, CA) using Lipofectamine 2000 (Invitrogen; Carlsbad, CA) as per the manufacturer's recommendations. Firefly luciferase readings were normalized against constitutive Renilla luciferase readings.

Western Blot Analysis

Treated MCF7 cells were washed with PBS, trypsinized and centrifuged at 5000 g. The pellets were washed with PBS and homogenized in M-PER tissue protein extraction reagent (Thermo Scientific; Waltham, MA) supplemented with protease and phosphatase inhibitors. Denatured proteins (5 µg) were separated in 10% or 12.5% SDS-PAGE gels, transferred to a PVDF membrane (Millipore; Billerica, MA) and incubated with antibodies to p53 (1:1000, Thermo Scientific; Waltham, MA) or MDM2 (1:1000, Santa Cruz; Dallas, TX). β-actin was used as a gel loading control for the whole cell homogenates. The blots were developed with enhanced chemiluminescence (ECL Clarity kit, Bio-Rad; Hercules, CA). Bands were visualized on a PVDF membrane and analyzed by LabWorks 4.5 software on a UVP Bioimaging System (Upland, CA). Quantification of results was performed by densitometry and the results analyzed as total integrated densitometric values (arbitrary units).

Co-Immunoprecipitation

Co-Immunoprecipitation (Co-IP) in cell homogenates was performed for p53 and MDM2 using “Catch and Release” immunoprecipitation kit (Millipore; Billerica, MA) according to the manufacturer's protocol. Briefly, 3×10^6 MCF7 cells were homogenized in Mammalian Protein Extraction Reagent (MPER) supplemented with protease and phosphatase inhibitors (Thermo Scientific; Waltham, MA). The homogenates containing the equivalent to 500µg of total protein content were incubated with 2µg of p53 mouse antibody (1:1000; Thermo Scientific; Waltham, MA) or 2µg of MDM2 mouse antibody (1:1000; Santa Cruz; Dallas, TX) overnight in the spin columns followed by elution. Eluate from p53 antibody precipitated protein-antibody complex (5µL) was resolved on a SDS-PAGE gel transferre onto a polyvinylidene difluoride (PVDF) membrane (BioRad; Hercules, CA) and incubated with MDM2 antibody followed by development with enhanced chemiluminescence ECL clarity (Bio-Rad; Hercules, CA). Analogously, eluate from anti-MDM2 antibody precipitated protein-antibody complex (5µL) was resolved on a SDS-PAGE gel transferred onto a PVDF membrane and incubated with p53 antibody followed by development with enhanced chemiluminescence ECL clarity. Bands were visualized on a PVDF membrane and analyzed by LabWorks 4.5 software on a UVP Bioimaging System.

Double Immunofluorescence Staining

Coverslip seeded cells were rinsed with PBS and fixed in cold acetone, blocked with 10% normal goat serum and incubated overnight at 4˚C with p53 antibody (anti-rabbit) and MDM2 antibody (anti-mouse). p53 was conjugated to Texas Red

and MDM2 to Alexa Fluor 488. All coverslips were washed and mounted with Vectashield containing DAPI and visualized with a Zeiss LSM 510 META confocal system coupled to a Zeiss Axiophot 200 inverted epifluorescence microscope (Carl Zeiss Microscopy; Dublin, CA). Quantification of percent overlap was determined using MetaMorph Microscopy Automation and Image Analysis Software (Molecular Devices).

Small Interfering RNA

The cells were transfected with MDM2 siRNA using Lipofectamine 2000 (Invitrogen; Carlsbad, CA) and incubated for 48 hours, followed by their respective treatments. Control (scrambled) siRNA were obtained from Santa Cruz Biotechnologies (sc-37007; Dallas, TX) . The siRNA to MDM2 sense and antisense strands were: GCUUCGGAACAAGAGACCC and GGGUCUCUUGUUCCGAAGC (Santa Cruz; Dallas, TX).

Statistical Analysis

All the assays were carried out in triplicates. Groups were compared using unpaired t-tests and One-Way Analysis of Variance (One-Way ANOVA) followed by Tukey's post-hoc test to correct multiple comparisons. Statistical analyses were performed with GraphPad Prism software 4.01. Quantitative data for all experimental analyses are presented as mean values ± S.E.M with unit value assigned to control and the magnitude of differences among the samples being expressed relative to the unit value of control.

Results

27-OHC Increases Proliferation in ER+ Breast Cancer Cells

27-OHC has been reported to be a novel SERM and an agent that can promote ER+ breast cancer growth (DuSell et al. 2008; Cruz, Torres, María E. Ramírez, et al. 2010; Nelson et al. 2013; Wu et al. 2013). As cell proliferation is considered a hallmark of tumor growth and cancer progression (Gao et al. 2005), we measured proliferation with and without 27-OHC treatment in ER-positive MCF7 and ER-negative MDA MB 231 cell lines. Treatment of MCF7 with 0.1 or 1μM 27-OHC increased cell proliferation by about 80% compared to treatment with vehicle (Fig. 9a). Treatment with estradiol of MCF7 cells also increased proliferation in a magnitude comparable to that of 27-OHC. On the other hand, the ER-negative cells MDA MB 231 did not exhibit significant proliferation when treated with 27-OHC (Fig. 9b). These result are in accordance with the recent discovery that 27-OHC binds to ER and exacerbates ER-positive breast cancers (Umetani and Shaul 2011; Wu et al. 2013).

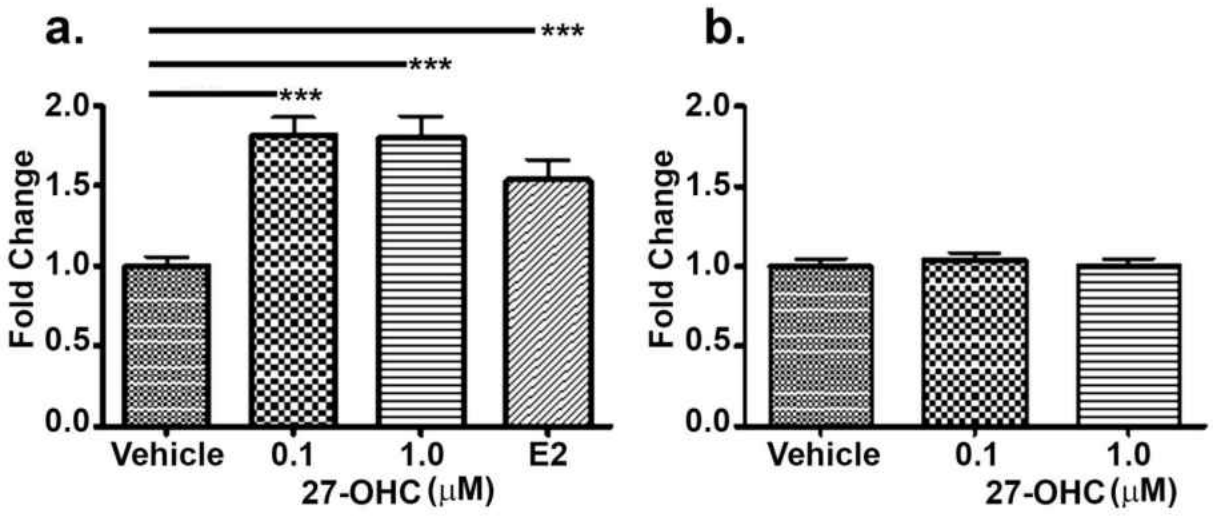


Figure. 9: 27-OHC induces proliferation in breast cancer cell lines. (a) Proliferation assay in the MCF7 cells shows an increase in proliferation 48h after treatment with 0.1 or 1μM of 27-OHC and 2nM Estradiol (E2) compared to treatment with vehicle. (b) In contrast to MCF7 cells, the MDA MB 231 cells exhibit no change in proliferation in the presence of 27-OHC either at 0.1 or 1μM concentration for 48h. Data is expressed as Mean ± S.E.M. ***$p<0.001$ versus vehicle

27-OHC Reduces Transcriptional Activity of p53

p53 is often mutated in breast cancers, thus implicating disruption of this gene in breast cancer progression. Moreover, p53 is a promising target in breast cancers (Sørlie et al. 2001; Walerych et al. 2012). MCF7 is one of the few breast cancer cells which has wild type p53, most ER+ breast cancer cell lines have mutated p53 (Okumura et al. 2002; Wasielewski et al. 2006). We used the MCF7 cell line

which is a commonly used cell model for breast cancer therapeutics studies (Comşa et al. 2015; Lee et al. 2015). Published work suggests that 27-OHC stimulates cell proliferation in breast cancer cells but not in normal breast epithelial cells (Cruz, Torres, María E. Ramírez, et al. 2010; Nelson et al. 2013; Wu et al. 2013). Since MCF7 cells express wild type p53, it is vital to examine the impact of 27-OHC on p53 activity (Okumura et al. 2002; Zheng et al. 2004; Wasielewski et al. 2006; Lim et al. 2009). Subsequently, we transfected cells with a luciferase reporter linked to a p53 receptor element and treated with 27-OHC. Treatment of MCF7 cells with 0.1 or 1μM 27-OHC significantly decreased p53-driven transcription by ~25% compared to incubation with vehicle (Fig. 10a). Interestingly, treatment with estradiol did not induce changes in p53 activity compared to vehicle (Fig. 10a). In the ER-negative MDA MB 231 cells, 27-OHC, either at 0.1 or 1μM, exerted no detectable effect on p53 activity (Fig. 10b). To test whether the 27-OHC effect on p53 mediated transcription was via ER, we used fulvestrant, an ER inhibitor. We found that co-treatment with fulvestrant attenuated the 27-OHC induced p53 inactivation (Fig. 10c). This result suggests that inhibitory effect of 27-OHC on p53 is ER mediated.

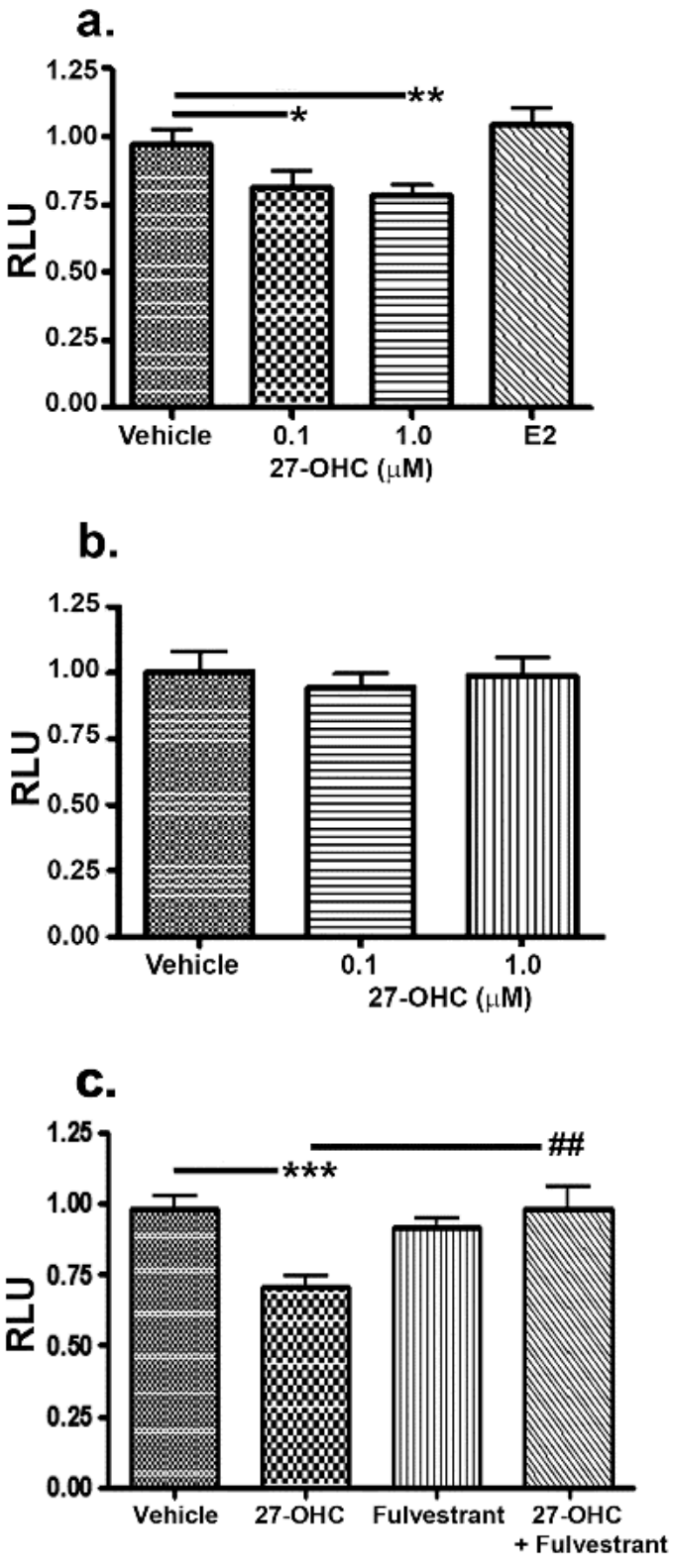

a.
1.25
1.00
0.75
0.50
0.25
0.00
RLU
*
**
Vehicle
0.1
1.0
E2
27-OHC (μM)
b.
1.25
1.00
0.75
0.50
0.25
0.00
RLU
Vehicle
0.1
1.0
27-OHC (μM)
c.
1.25
1.00
0.75
0.50
0.25
0.00
RLU
***
##
Vehicle
27-OHC
Fulvestrant
27-OHC
+ Fulvestrant

Figure 10: 27-OHC reduces p53 activation. (a) Luciferase reporter assay in MCF7 cells showed lower p53 activity in the presence of 0.1μM 27-OHC, 1μM 27-OHC, or 2nM Estradiol (E2) for 24h. (b) No change in p53 activity was detected with the luciferase reporter assay in the ER-negative MDA MB 231 cells treated with 0.1μM or 1μM 27-OHC for 24h. (c) Luciferase reporter assay in MCF7 cells demonstated that fulvestrant, an ER inhibitor, attenuates 27-OHC induced p53 inactivity when treated with 1μM 27-OHC and/or 5μM of fulvestrant. Data is expressed as Mean ± S.E.M. *$p<0.05$, **$p<0.01$, ***$p<0.001$ versus vehicle.$^{\#\#}p<0.01$ versus 27-OHC only. RLU=Relative Luciferase Units

27-OHC Regulates p53 and MDM2 Expression

Regulation of p53 degradation is important to maintain its activity. MDM2 plays a critical role in regulating p53 levels by enhancing p53 degradation. MDM2 catalyzes p53 degradation by flagging it for destruction. In contrast, during DNA damage MDM2 undergoes self-ubiquitination and downregulates its own expression, which leads to the upregulation of the DNA damage response through p53 (Moll and Petrenko 2003; Manfredi 2010). MDM2 is overexpressed in human cancers where it causes the disruption of p53 signaling and potentially other oncogenic pathways (Moll and Petrenko 2003; Manfredi 2010). We determined the effect of 27-OHC on p53 and MDM2 expression levels using western blot analyses. We found that treatment of cells with 27-OHC decreased p53 expression levels by ~50% and increased MDM2 expression levels by ~28% compared to vehicle (Fig. 11a-c). This result suggests that 27-OHC downregulates p53 expression and upregulates MDM2 expression.

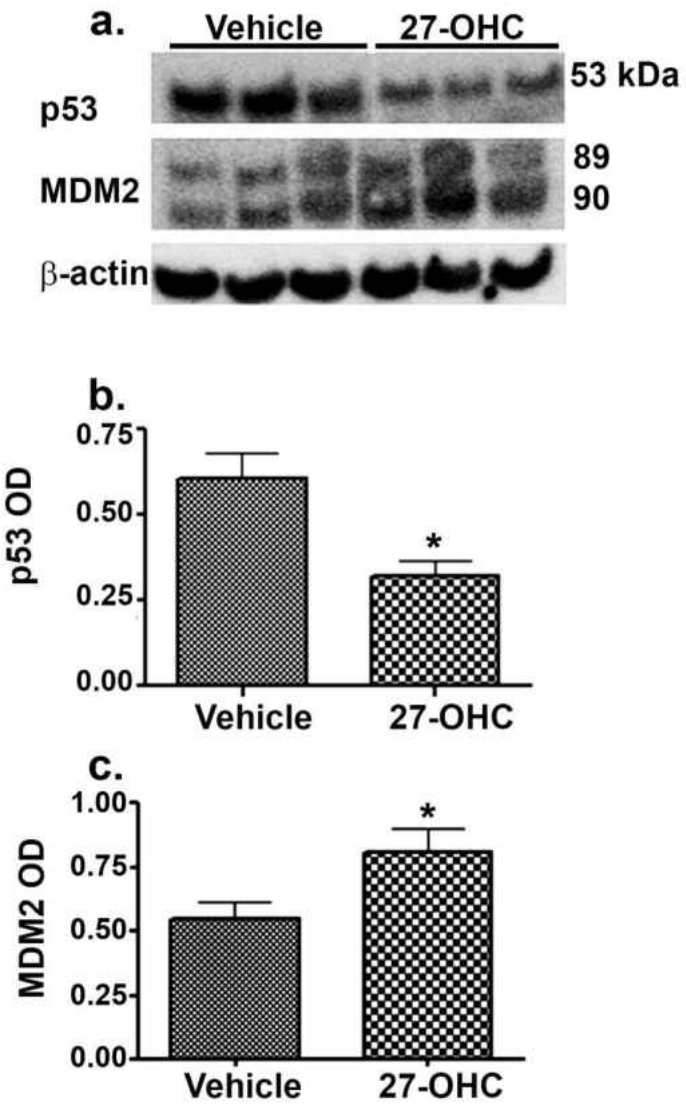
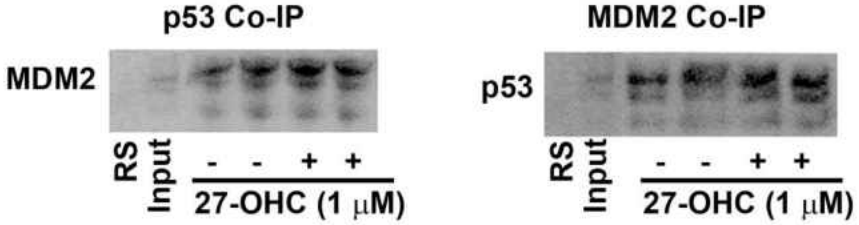


Figure 11: 27-OHC reduces p53 and increases MDM2 levels. (a) Representative western blot and densitometric analysis of MCF7 cells showing a substantial decrease in p53 levels (b) and a significant increase in MDM2 levels (c) following treatment with 1μM of 27-OHC for 24h. Data is expressed as Mean ± S.E.M. *p<0.05 versus vehicle

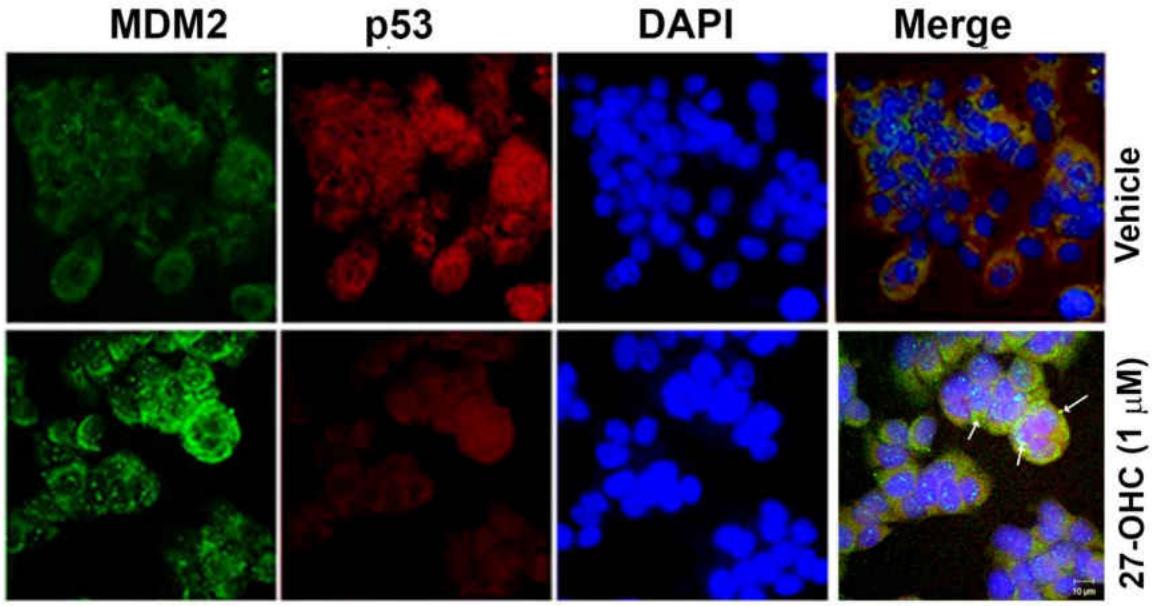
27-OHC Enhances p53 and MDM2 Dimerization

The relationship between p53 and MDM2 has been widely studied. MDM2 is known to bind to p53 and shuttle it to the cytoplasm for degradation (Alarcon-Vargas 2002; Moll and Petrenko 2003). To determine whether 27-OHC enhances the interaction between MDM2 and p53, we performed a co-immunoprecipitation assay. We found that treatment with 27-OHC increased MDM2 and p53 binding. When MDM2 was blotted against p53 immuno-precipitated lysate or when p53 was blotted against MDM2 immuno- precipitated lysate, an increase in staining for the other protein was observed (Fig. 12a). We also used double immunofluorescence staining to observe p53-MDM2 co-localization. Subsequently, we found that 27-OHC treatment, caused a significant increase in overlap of labelled p53 and MDM2 at the nuclear envelope (Fig. 12b,c). This indicates that 27- OHC enhances the physical interaction between p53 and MDM2.

a.


p53 Co-IP
MDM2
RS Input
- - + +
27-OHC (1 μM)
MDM2 Co-IP
p53
RS Input
- - + +
27-OHC (1 μM)

b.


MDM2
p53
DAPI
Merge
Vehicle
27-OHC (1 μM)

c.

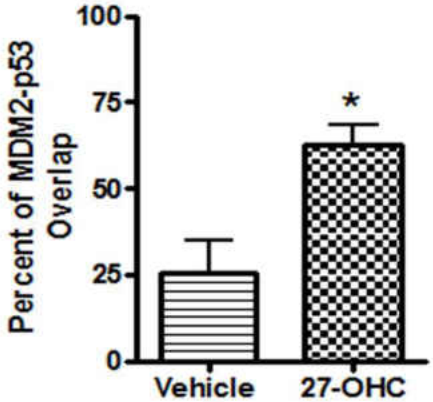

Percent of MDM2-p53 Overlap
100
75
50
25
0
*
Vehicle
27-OHC

Figure 12: 27-OHC promotes p53-MDM2 interaction in MCF7 cells. (a) Representative non-denatured western blots for p53 IP blotted against MDM2 and MDM2 IP blotted against p53 demonstrating an increase in p53-MDM2 binding in of MCF7 cells treated for 24h as follows: lane 1 Rabbit Serum (negative control); lane 2: lysate only; lane 3, 4: vehicle only; lane 5,6: 1µM 27-OHC. (b) Representative confocal microscopy images showing increased intensity of MDM2 staining (green) and reduced intensity of p53 staining (red) following treatment of MCF7 cells with1µM 27-OHC. Arrows indicate co-localization of p53 and MDM2 in MCF7 cells treated with 1µM 27-OHC for 24h. Bar, 10µm. (c) Representative graph showing increased percent of overlap between MDM2 and p53 in the confocal images. Data is expressed as Mean ± S.E.M. *$p<0.05$ versus vehicle

27-OHC Increased Proliferation via MDM2 Mediated p53 Inactivity

To determine whether 27-OHC promotes cell proliferation via p53 inactivation; we treated cells with the p53 activator, Tenovin-1 (Lain et al. 2008). We found that Tenovin-1 significantly increased p53 transcriptional activity (Fig 13a) and inhibited 27-OHC-induced proliferation (Fig. 13b). This suggests that p53 inactivation is necessary for 27-OHC induced cell proliferation. Given that 27-OHC treatment increased MDM2 levels, enhanced MDM2-p53 binding and decreased p53 levels (Fig. 11&12), we tested whether if 27-OHC-induced cell proliferation was due to MDM2 dependent p53 degradation. We treated cells with the MDM2-p53 interaction inhibitor, Nutlin-3 (Park et al. 2013). We found that Nutlin-3 blocked 27-OHC-induced cell proliferation comparable to that of basal

levels (Fig. 13c). This result suggests that MDM2-p53 binding is required for 27-OHC induced cell proliferation. To investigate if MDM2 expression is specifically necessary for 27-OHC induced cell proliferation, we knocked down MDM2 expression using siRNA. Inhibition of MDM2 blocked 27-OHC induced cell proliferation (Fig. 13d,e). This data strongly suggests that 27-OHC requires MDM2-mediated p53 degradation to induce cell proliferation.

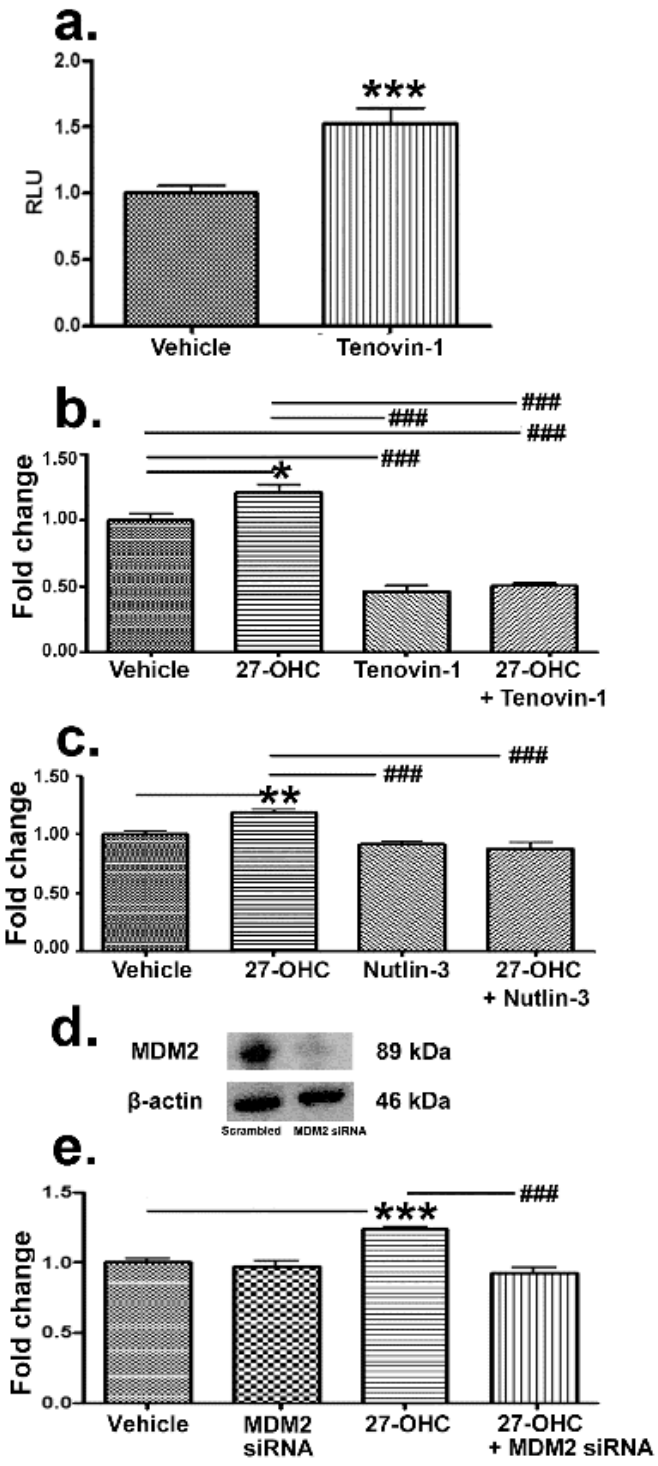


Figure 13: 27-OHC induces proliferation via MDM2-mediated p53 inactivation. (a) Luciferase reporter assay demonstrates an increase in p53 activity in MCF7 in the presence of 10μM Tenovin-1 for 48h. (b) Cell proliferation assay

demonstrates that 27-OHC-induced proliferation is attenuated by Tenovin-1 in MCF7 cells. Cells were treated vehicle, 1μM 27-OHC, 10μM Tenovin-1, or 1μM 27-OHC + 10μM Tenovin-1 for 48h. (c) In MCF7 cells, 1μM 27-OHC-induced proliferation is also attenuated by the MDM2-p53 interaction inhibitor Nutlin-3. Cells were treated with vehicle, 1μM 27-OHC, 5μM Nutlin-3, or 1μM 27-OHC + 5μM Nutlin-3 for 48h. (d) Representative blot demonstrates MDM2 knock down efficiency in MCF7. (e) 1μM 27-OHC-induced proliferation is also attenuated by MDM2 siRNA. Cells were incubated with respective treatments for 48h. Data is expressed as Mean ± S.E.M. versus vehicle. Data is expressed as Mean ± S.E.M. *$p<0.05$, **$p<0.01$, ***$p<0.001$ versus vehicle, and $^{###}$ $p<0.001$ versus 27-OHC only. RLU=Relative Luciferase Units

Discussion

The goal of this study was to elucidate cellular mechanisms involved in 27-OHC-induced proliferation in breast cancer cells. Our results demonstrate that 27-OHC decrease p53 activity and protein levels, which results in increased cell proliferation. Our study also shows that 27-OHC increases MDM2 levels and enhances the interaction between p53 and MDM2. We further show that 27-OHC-induced proliferation is dependent on MDM2-mediated p53 degradation. Our results are the first showing that 27-OHC acting through ER, exacerbates breast cancer cell proliferation via the p53-MDM2 axis.

P53 is a highly regulated protein in the cell and has a short half-life (Giaccia and Kastan 1998). Loss of p53 function and/or perturbations in its signaling pathways via mutations plays an important role in several cancers

(Hanahan and Weinberg 2000; Sherr and McCormick 2002; SHERR 2004). p53 plays an important role in “guarding the genome” from genotoxic stress and regulates apoptosis, cell cycle, senescence and metabolism. Lack of functional p53 leads to abnormalities in cell cycle and apoptosis, which may lead to cancer progression. Approximately, 23% of breast cancers exhibit a p53 mutation and loss of p53 function via mutation remains one of the main molecular characteristics of breast carcinomas, while more spontaneous cancers such as ovarian, intestinal, and lung cancers have higher incidences of p53 mutations (Walerych et al. 2012; Yu et al. 2014 Apr 23). In this report, we show that the cholesterol oxidation metabolite 27-OHC dysregulates p53 expression and function.

We report that 27-OHC, a SERM, inhibited wild type p53 activity, while interestingly, estradiol, a standard and endogenous ligand for ER had no effect on p53 activity. This demonstrates that 27-OHC may contribute to ER-positive breast cancer progression via different mechanisms compared to known estrogens. To verify that 27-OHC exerted it effects via ER, we treated cells with fulvestrant, an ER inhibitor, concomitantly with 27-OHC and found that it blocked the p53 transcriptional inactivation effects of 27-OHC. This demonstrates that while 27-OHC and estradiol activate the same receptor (ER), the downstream events appear to be distinct. Regulation of estrogen levels in humans has been extensively studied and is a primary target for therapy using interventions such as aromatase inhibitors and ovarian ablation (Gonzalez-Angulo et al. 2007). Although hormone therapy is relatively effective, such therapy is challenged by

endocrine resistance and recurrence of breast cancers. Hormone therapy is designed to specifically reduce estrogen levels (Hasson et al. 2013; Jankowitz and Davidson 2013). The recent discovery that 27-OHC also activates ER and promotes ER-positive breast cancer progression, may explain why hormone therapy may not be as effective as expected. Furthermore, a recent study by Nelson and colleagues demonstrated that the gene expression profile in MCF7 breast cancer cells grown in the presence of 27-OHC was remarkably different relative to estradiol-treated MCF7 cells, with 788 unique genes in the 27-OHC profile that are different from the estradiol profile of 8141 genes (Nelson et al. 2013). In the above mentioned study, 27-OHC and estradiol regulated 1511 shared genes (Nelson et al. 2013). Understanding the role of 27-OHC in ER-positive breast cancer may reveal novel molecular mechanisms that play a role in breast cancer progression, resistance and recurrence of breast cancers.

There is a positive correlation between cholesterol and 27-OHC levels in humans. Patients with hypercholesterolemia have higher levels of 27-OHC and thus may be at a greater risk for developing ER-positive breast cancer (Nelson et al. 2013). 27-OHC is synthesized from cholesterol through hydroxylation by a cytochrome P-450 enzyme, sterol 27 hydroxylase (CYP27A1), localized in the inner mitochondrial membrane of the liver. 27-OHC is a substrate for bile acid synthesis, and when bile acid levels are adequate, excess levels of 27-OHC are catabolized by CYP7B1 (Fu et al. 2001; Ma et al. 2014 May 1). In support of hypercholesterolemia as a risk factor for developing breast cancers, the use of cholesterol lowering agents, such as HMG-CoA reductase inhibitors (statins),

has been associated with better prognosis and breast cancer survival rates (Ahern et al. 2011). Furthermore, Cruz et al showed that in ER-positive mammary tumor cells, simvastatin blocked 27-OHC induced cell proliferation (Cruz, Torres, María E. Ramírez, et al. 2010).

Our results add further insights into the potential molecular mechanisms by which 27-OHC influences cancer cell progression. We show that 27-OHC increases levels of the E3 ubiquitin ligase MDM2 and its interaction with p53. MDM2 is known to regulate p53 activity by flagging it for ubiquitination and proteasomal degradation (Alarcon-Vargas 2002). Since DNA damage is a potent activator of p53, prior to DNA damage MDM2 binds to both p53 and ribosomal protein RPL26 resulting in ubiquitination and proteasomal degradation. Upon DNA damage, p53 and MDM2 undergo posttranslational modifications, inhibiting their interaction and subsequently activating p53 activity (Moll and Petrenko 2003; Khoo et al. 2014). In the presence of 27-OHC, MDM2 and p53 interaction is enhanced, resulting in p53 inactivation and degradation. It is suggested that MDM2 by itself may be an oncogene (Momand 1998; Wang et al. 1999). MDM2 is overexpressed in various cancers including sarcoma, leukemia, breast cancers, melanoma, and glioblastoma (Jones et al. 1998; Momand 1998).

We also demonstrate that 27-OHC-induced cell proliferation in the ER-positive breast cancer MCF7 cells is dependent on the inactivation of p53. Treatment with Tenovin-1, a potent p53 activator, attenuated 27-OHC-induced cell proliferation. This supports the notion that 27-OHC induced p53 inactivation promotes breast cancer cell proliferation. Since p53 can undergo inactivation and

degradation through multiple mechanisms (Moll and Petrenko 2003; Kruse and Gu 2009; Tsvetkov et al. 2010), it was vital to test the role of MDM2 specifically in 27-OHC induced p53 inactivation that led to cell proliferation. To verify whether p53 inactivation is MDM2-dependent, we treated cells with Nutlin-3, an MDM2-p53 interaction inhibitor (Miyachi et al. 2009; Poyurovsky et al. 2010). We found that Nutlin-3 prevents MDM2 mediated and 27-OHC-induced degradation of p53. We also found that upon knocking down MDM2, 27-OHC induced cell proliferation was attenuated, suggesting that inactivation of p53 is MDM2 dependent. This data indicates that 27-OHC exacerbates ER-positive breast cancer progression by inactivating and degrading p53 via MDM2.

Although the prognostic value of p53 in ER-positive breast cancers is controversial, we propose that in the presence of excess 27-OHC, p53 function is compromised via MDM2, causing an exacerbation of ER-positive breast cancer progression. Subsequently, activation of p53 has been considered to be an important therapeutic target in tumorigenesis and cancer progression. A number of small molecules have been developed to activate wild type p53 and reactivate mutant p53. Interestingly, both DNA based and dendritic cell delivered p53 vaccines have been developed to activate and upregulate p53 (van der Burg et al. 2001; Speetjens et al. 2009). In addition, gene therapy has been used against breast carcinomas (Obermiller et al. 2000; Sasaki et al. 2001; Song and Boyce 2001; Rejeeth and Kannan 2014 May 4). Recently, Rejeeth et al. (2014) used silica nanoparticle supplemented with transferrin to administer p53 to MCF-7 and showed that such a treatment reduced cell growth by 60.7%.

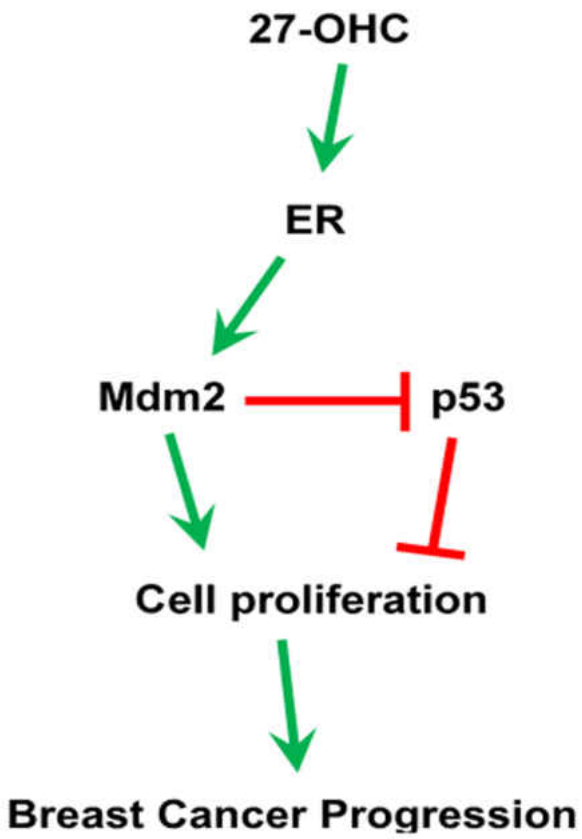


Figure 14: Proposed mechanism of action of 27-OHC in ER+ breast cancer. 27-OHC activates ER, which upregulates MDM2 and mediates p53 inactivation and degradation resulting in increased in cell proliferation. Thus, exacerbating ER+ breast cancer progression. Activation is denoted by *arrows*; inhibition is indicated by bars

In summary, we demonstrate that 27-OHC exacerbates ER-positive MCF7 cancer cell proliferation by disrupting p53 activity. We propose that 27-OHC activates ER, an effect that leads to activation of MDM2 and subsequent inactivation of p53. Fulvestrant, an ER specific antagonist, blocks 27-OHC activation of ER, resulting in disinhibition of Mdm2-mediated p53 transcriptional activity. It has been shown that ER activation enhances MDM2 expression and

that fulvestrant attenuates MDM2 upgregulation by inhibiting ER (Dolfi et al. 2014). Since ER upregulates MDM2, which in turn inactivates p53, we asked if fulvestrant would inhibit MDM2-mediated p53 inactivation. Indeed, when we measured p53 activity, we found that fulvestrant inhibited 27-OHC-induced Mdm2-mediated p53 inactivation (Fig.10). Our data indicates that 27-OHC acting through ER can disrupt the p53 response. We suggest that individuals with high levels of 27-OHC may have an increased risk of developing ER-positive breast cancers via loss of constitutive p53 activity. We also demonstrate that 27-OHC enhances MDM2-p53 interaction resulting in loss of p53 expression and activity. Such results strongly suggest an MDM2 involvement in the 27-OHC-induced p53 inactivity and subsequent cell proliferation (Fig. 14). Our findings provide a novel molecular mechanism for 27-OHC that may contribute in the pathophysiology of ER-positive breast cancers. Understanding the MDM2-p53 interplay in the presence of 27-OHC may reveal innovative therapeutic avenues that can inhibit ER-positive breast cancer progression.

CHAPTER III

ROLE OF 27-HYDROXYCHOLESTEROL IN PROSTATIC HYPERPLASIA

27-hydroxycholesterol Stimulates Cell Proliferation and Resistance to Docetaxel-induced Apoptosis in Prostate Epithelial Cells

Abstract

Although the causes of prostate cancer (PCa) and benign prostatic hyperplasia (BPH) are not known, the role of oxidative stress, aging, and diet are suspected to increase the incidence of prostate complications. The cholesterol oxidation derivative (oxysterol) 27-hydroxycholesterol (27-OHC) is the most prevalent cholesterol metabolite in the blood. As aging, oxidative stress, and hypercholesterolemia are associated with increased risk of PCa and BPH, and because 27-OHC levels also increased with age, hypercholesterolemia and oxidative stress, determining the role of 27-OHC in the progression of PCa and BPH is warranted. In this study, we determined the effect of 27-OHC in human prostate epithelial cells RWPE-1. We found that 27-OHC stimulates proliferation and increases androgen receptor (AR) transcriptional activity. 27-OHC also increased PSA expression and enhanced AR binding to the androgen response element compared to controls. Silencing AR expression with siRNA markedly reduced the 27-OHC-induced proliferation. Furthermore, 27-OHC blocked docetaxel-induced apoptosis. Altogether, our results suggest that 27-OHC may

play an important role in PCa and BPH progression by promoting proliferation and suppressing apoptosis.

Introduction

Prostate health is an area of growing concern. Over 14% of men will develop prostate cancer (PCa) and greater than 70% of men will develop benign prostatic hyperplasia (BPH) by the age of 70 (Guess et al. 1990; Delongchamps et al. 2006). The etiologies of PCa and BPH are unknown but environmental factors including diet are suggested to play a role in the progression of these pathologies. Aging, genetic susceptibility, obesity, low physical activity, androgens, and inflammatory conditions are all associated with both PCa and BPH (Chokkalingam et al. 2003; Ørsted and Bojesen 2013).

Cholesterol involvement in PCa has been suspected for over a century when it was found to accumulate in PCa cells (White 1909). More recent studies demonstrated a positive correlation between high plasma cholesterol and high-grade PCa (Mondul et al. 2010). Moreover, some studies reported a reduction in the risk of high-grade PCa (Breau et al. 2010; Papadopoulos et al. 2011) and BPH (Lee et al. 2013) with the cholesterol-lowering statins. However, the efficacy of lowering cholesterol levels currently is not proven and whether increased cholesterol levels are causal factors of PCa and BPH is still unknown.

Several lines of evidence suggest that cholesterol oxidation products, not cholesterol *per se,* may increase the risk of PCa and BPH risk. 27-OHC is the main oxysterol in the circulation, and its levels can be increased by aging, oxidative stress, and hypercholesterolemia (Hirayama et al. 2009; Nelson et al. 2013), factors that are all suspected to increase the risk for PCa and BPH. Evidence suggests that 27-OHC can be deleterious as it can act as a SERM (DuSell et al. 2008; Umetani and Shaul 2011) and a liver X receptor (LXR) ligand (Fu et al. 2001). Both ER and LXR are involved in steroid signaling pathways and influence inflammation, cell proliferation, and many other metabolic processes. However, the extent to which 27-OHC also regulates androgen receptors (AR) which play a central role in the pathogenesis of BPH and PCa is yet to be determined. In this study we determined the effects of 27-OHC on cell proliferation and the role of AR in 27-OHC induced cell proliferation.

We found that 27-OHC increases cell proliferation and AR transcriptional activity. We also demonstrate that 27-OHC enhances AR binding to the Prostate Specific Antigen (PSA) promoter and increases PSA expression. To investigate whether AR is required in 27-OHC-induced cell proliferation, we silenced AR gene expression using siRNA and found that 27-OHC induced cell proliferation is AR dependent. Additionally, to determine whether 27-OHC contributes to apoptosis-resistance, we treated cells with 27-OHC and docetaxel and found that 27-OHC inhibits the pro apoptotic effects of docetaxel by visualizing nuclear fragmentation using TUNEL assay. Overall, our data supports the notion that 27-

OHC induces cell proliferation in an AR-dependent manner and that 27-OHC exhibits anti-apoptotic activities in prostate cells.

Methods

Reagents

27-OHC was purchased from Santa Cruz Biotechnologies (Dallas, TX), docetaxel and fulvestrant from Cayman Chemicals (Ann Arbor, MI), β-estradiol from Sigma-Aldrich (St. Louis, MO) and the reporter constructs encoding androgen receptor response elements conjugated to the firefly luciferase gene from SA Biosciences (Valencia, CA). All cell culture reagents, with the exception of fetal bovine serum (FBS) (Atlanta Biologicals; Flowery Branch, GA) were from Invitrogen. Human RWPE-1 cells were purchased from ATCC (Manassas, VA). Concentrations of solvent in treatments was less than 0.1%.

Cell Culture

Non-tumorigenic human prostate epithelial RWPE-1 cells were maintained in Keratinocyte serum free medium (Invitrogen; Carlsbad, CA) supplemented with 0.05mg/ml BPE and 5ng/ml EGF. Cells were supplemented with 100U/ml penicillin, 100µg/ml streptomycin 0.25µg/ml amphotericin (Sigma; St.Louis, MO) and cultured at 5% CO_2 and 37˚C. Cells were treated with vehicle (ethanol in media), 0.1 µM or 1µM of 27-OHC. Stock solutions of 27-OHC were prepared in 100% ethanol and stored at -80°C. 27-OHC stock solution was dissolved in appropriate volumes of media to prepare the working solutions of 0.1 µM or 1µM.

Our study was approved by the Institutional Biosafety Committee at the University of North Dakota.

Cell Proliferation Assays

To determine the effects of 27-OHC on cell proliferation, we treated cells with 27-OHC at concentrations that have been shown to promote breast cancer progression (Cruz, Torres, María E. Ramírez, et al. 2010; Nelson et al. 2013; Wu et al. 2013). Proliferation assays were conducted on black 96 well plates using CyQUANT Direct Cell Proliferation Assay (Invitrogen; Carlsbad, CA) which quantifies cell number using DNA content and membrane integrity. Cells seeded at $5x10^3$ cells/well were treated with vehicle, 0.1 µM or 1µM of 27-OHC and incubated for 48 hours. Cells were then stained as per the manufacturer's protocol and read using Spectra MAX GEMINI EM (Molecular Devices; Sunnyvale, CA).

Metabolic Activity Assay

Cell metabolic activity was quantified by measurement of the reduction of MTS to formazan product using CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega; Madison, WI) according to the manufacturer's protocol. The assay of the formation of formazan was performed by measuring absorbance change using a microplate reader (Spectromax plus; Molecular Devices; Sunnyvale, CA) 48 h after treatments.

Dual Luciferase Assays

Dual luciferase assays were conducted on white 96 well plates using the Dual Luciferase Reporter Assay System (Promega; Madison, WI). Cells were incubated for 18 hours with transfection ready AR response element conjugated with firefly luciferase construct and constitutively expressing Renilla Luciferase construct using Lipofectamine 2000 (Invitrogen; Carlsbad, CA) as per the manufacturer's protocol. Following transfection cells were treated with 0 (vehicle), 0.1 or 1μM 27-OHC for 24 hours. After 24 hours of treatment, cells were lysed and measured in Relative Luciferase Units (RLU) as per the manufacturer's protocol. Firefly luciferase readings were normalized against constitutive Renilla luciferase readings.

Chromatin Immunoprecipitation (ChIP) Analysis

ChIP analysis was performed to evaluate the extent of AR binding to the DNA elements in the androgen receptor elements (ARE) regions respectively using SimpleChIP™ Enzymatic Chromatin IP kit (Cell Signaling; Beverly, MA). Briefly, cells from each treatment group (1×10^7 cells) were washed with PBS, trypsinized, and centrifuged at 5000 g. The pellet containing the cells was further washed with PBS and cross-linked using 37% formaldehyde for 15 min followed by the addition of glycine solution to cease the cross-linking reaction. The cells were washed with 4x volumes of 1x PBS and centrifuged at ~300g for 5 min. The pellet was re-suspended and incubated for 10 min in 5 ml of cell lysis buffer containing DTT and protease and phosphatase inhibitors. The cross-linked chromatin from each sample was apportioned into three equal parts. One third of

the cross-linked chromatin was set aside as “input”. One third of the cross-linked chromatin from each sample was incubated with 5 µg of AR rabbit antibody (Active Motif), while the remaining one third of the cross-linked chromatin from each sample was incubated with 5 µg of normal Rabbit IgG to serve as negative control. The cross-linked chromatin samples were incubated overnight at 4°C with their respective antibodies. The DNA-protein complexes were collected using Protein G agarose beads. The samples were incubated with 2 µL of Proteinase K for 2 hours at 65°C. The crude DNA extract was eluted and then washed several times with wash buffer containing ethanol followed by purification with the DNA spin columns. The pure DNA was eluted out of the DNA spin columns using DNA elution buffer. The relative abundance of the AR antibody precipitated chromatin containing the AR binding site in the ARE region was determined by qPCR using an SYBR Green Mastermix kit following the manufacturer’s instructions (Invitrogen; Carlsbad, CA). ARE sequence on the PSA promoter F: 5′-TCTGCCTTTGTCCCCTAGAT-3′ and R: 5′-AACCTTCATTCCCCAGGACT-3′ (Horie-Inoue et al. 2004). The amplification was performed using Step NE plus PCR Detection System (Invitrogen; Carlsbad, CA). The fold enrichment was calculated using the $\Delta\Delta C_t$ method which normalizes ChIP C_t values of each sample to the % input and background.

Western Blot Analysis

Treated cells were washed with PBS, trypsinized, and centrifuged at 5000 g. The pellet was washed with PBS and homogenized in M-PER tissue protein extraction reagent (Thermo Scientific; Waltham, MA) supplemented with

protease and phosphatase inhibitors. Denatured proteins (5 µg) were separated in 10% or 12.5% SDS-PAGE gels, transferred to a PVDF membrane (Millipore) and incubated with antibodies to PSA (1:1000, Santa Cruz; Dallas,TX), or AR (1:1000, Santa Cruz; Dallas, TX). β-actin was used as a gel loading control. The blots were developed with enhanced chemiluminescence (ECL Clarity kit, Bio-Rad). Bands were visualized on a polyvinylidene difluoride membrane and analyzed by LabWorks 4.5 software on a UVP Bioimaging System. Quantification of results was performed by densitometry and the results analyzed as total integrated densitometric values (arbitrary units).

Small interfering RNA

The cells were transfected with AR siRNA using Lipofectamine 2000 (Invitrogen; Carlsbad, CA) and incubated for 24 hours, followed by their respective treatments. siRNA to AR sense and antisense strands were: 5'-CGGGAAGUUUAGAGAGCUATT-3',: 5'UAGCUCUCUAAACUUCCCGTG-3' (Hs_AR_5, SA Biosciences; Valencia, CA).

TUNEL Assay

The TUNEL assay was performed using DeadEnd Fluorometric TUNEL assay (Promega; Valencia, CA) for detection of apoptosis. The TUNEL staining was performed according to manufacturer's instructions. Cells were permeabilized with Triton-X, washed with PBS, and incubated with terminal deoxynucleotidyl transferase, fluorescein-12-dUTP. The fluorescein-12-dUTP labeled DNA was then visualized directly by fluorescence microscopy. DAPI was used as counter

stain for staining the nucleus. Slides were visualized using DMI 6000 (Leica Microsystems; Buffalo Grove, IL).

Statistical analysis

The significance of differences were assessed by unpaired t-test and One Way Analysis of Variance (One Way ANOVA) followed by Tukey's post-hoc test. Statistical analysis was performed with GraphPad Prism software 4.01. Quantitative data for experimental analysis are presented as mean values ± S.E.M with unit value assigned to control and the magnitude of differences among the samples being expressed relative to the unit value of control.

Results

27-OHC increases cell proliferation

We found that 27-OHC increases proliferation by ~60% at 0.1 µM and ~40% at 1.0 µM (Fig 15a). To confirm our results, we performed MTS assay to assess the mitochondrial activity of cells. We found that 27-OHC (0.1 and 1 µM) also increased the metabolic activity of the cells (Fig 15b). This data suggests that 27-OHC induces cell proliferation in prostate epithelial cells.

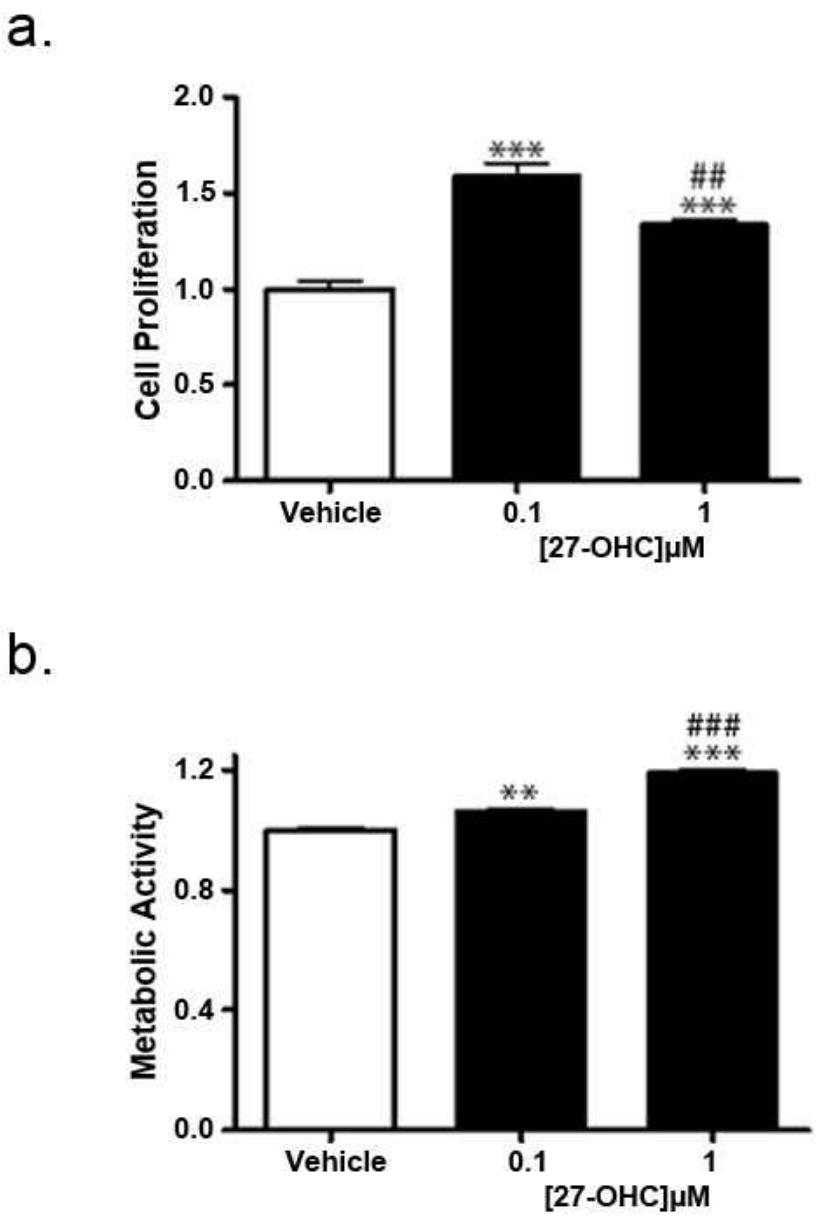


Figure 15: 27-OHC induces proliferation in prostate cells. Cell proliferation assay in RWPE-1 (a) cells demonstrates a significant increase in proliferation in the presence of 27-OHC (0.1 and 1μM). MTS assay shows a significant increase in cell metabolic activity in the presence of 27-OHC in RWPE-1 (b). Readings were recorded 48 hours after treatment with 27-OHC. Data is expressed as Mean ±

S.E.M. ***p<0.001 and **p<0.01 versus controls, ###p<0.001 and ##p<0.01 versus 0.1 μM 27-OHC treatment.

27-OHC increases AR transcriptional activity

We treated cells with 27-OHC and measured the transcriptional activity of AR using an ARE tagged luciferase reporter. We found that 27-OHC treatment significantly increased AR transcriptional activity (Fig. 16).

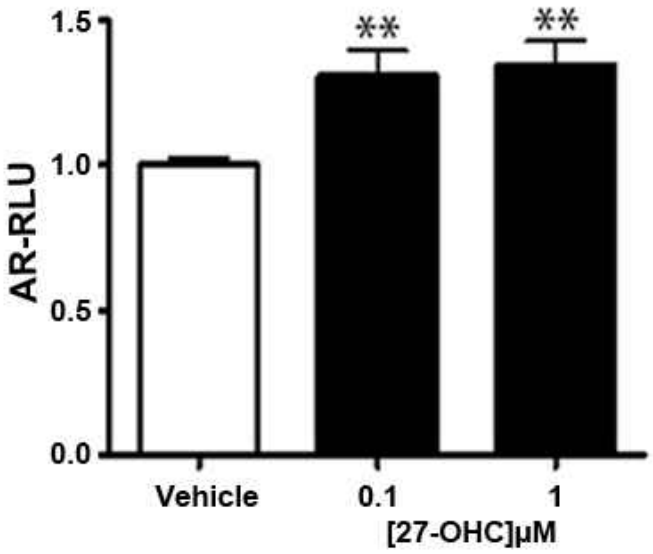


Figure 16: 27-OHC increases AR transcriptional activity. Luciferase reporter assay shows an increase in AR transcriptional activity in the presence of 27-OHC (0.1 and 1μM) in RWPE-1 cells. Readings were recorded 24 hours after treatment. Data is expressed as Mean ± S.E.M. **p<0.01 versus controls.

27-OHC increases PSA expression and AR binding to the PSA promoter

Using western blot analyses, we showed elevated levels of PSA in 27-OHC treated cells. PSA levels significantly increased with 0.1µM and 1.0µM of 27-OHC (Fig 17 a,b). To determine if the elevated levels of PSA were due to the increase in AR transcriptional activity, we performed a ChIP assay to analyze AR binding to the androgen receptor element (ARE) on the PSA promoter. We found an increase in AR binding to the ARE of the PSA promoter by ~17 fold when treated with 0.1µM of 27-OHC and ~11 fold when treated with 1µM of 27-OHC (Fig. 17c). This data suggests that 27-OHC enhances AR mediated PSA expression.

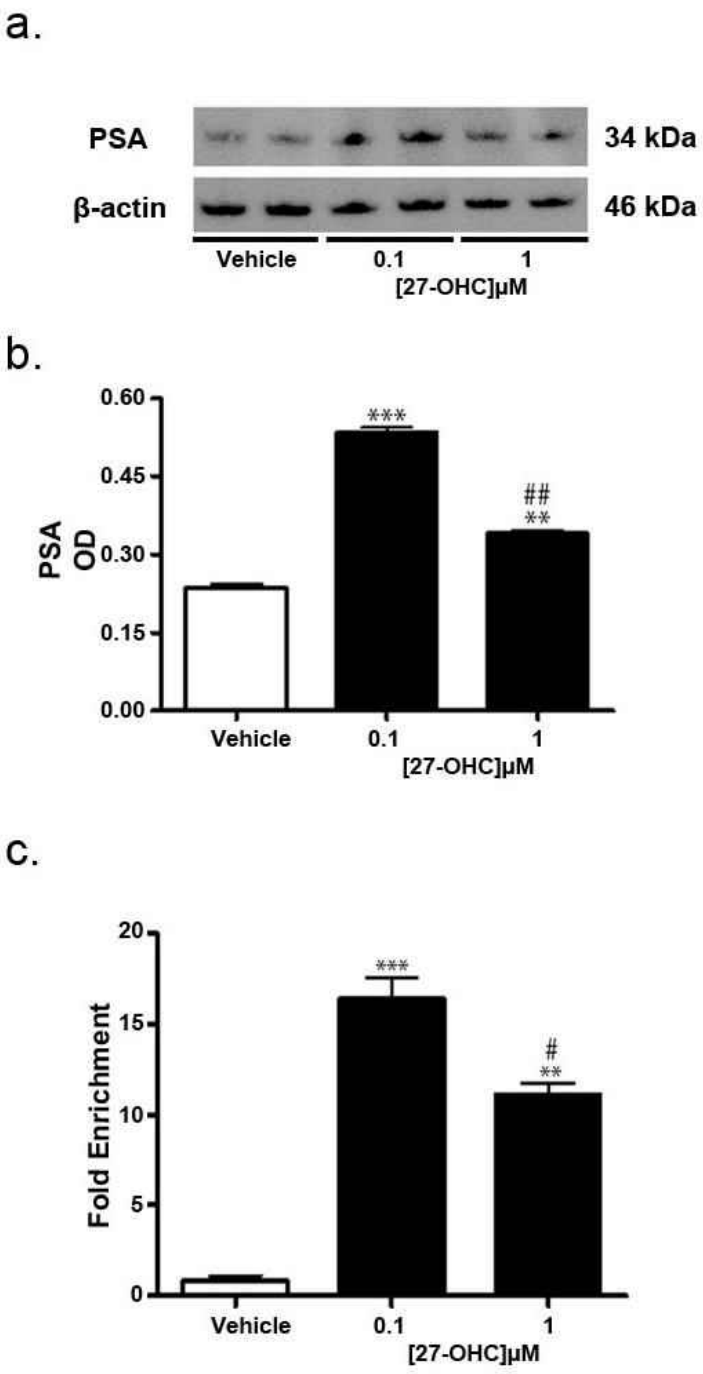


Figure 17: 27-OHC increases PSA protein levels via AR. Representative western blot (a) and densitometric analysis (b) showing a significant increase in PSA protein levels in RWPE-1 cells treated with 27-OHC (0.1 and 1μM) for 48 h. ChIP analysis of AR binding to the ARE on the PSA promoter shows an increase in AR

binding in the presence of 27-OHC that is higher with 0.1 than 1.0 μM concentrations (c). Data is expressed as Mean ± S.E.M. ***$p<0.001$ versus controls, ##$p<0.01$, and #$p<0.05$ versus 1μM 27-OHC treatment.

27-OHC-induced cell proliferation is dependent on AR

We found that siRNA to AR reduced the protein levels of AR in untreated cells (Fig. 18a) and diminished cell proliferation in 27-OHC-treated cells (Fig. 18b). This result suggests that 27-OHC-induced cell proliferation involves AR. As 27-OHC does not directly bind to AR and is an ER modulator (Nelson et al. 2011; Umetani and Shaul 2011; Wu et al. 2013), it was important to determine the role of ER in prostate cell proliferation. We found that fulvestrant, an ER inhibitor, when concomitantly treated with 27-OHC, attenuated 27-OHC induced cell proliferation (Fig 18c). This result suggests that ER may function in concert with AR to induce 27-OHC induced cell proliferation.

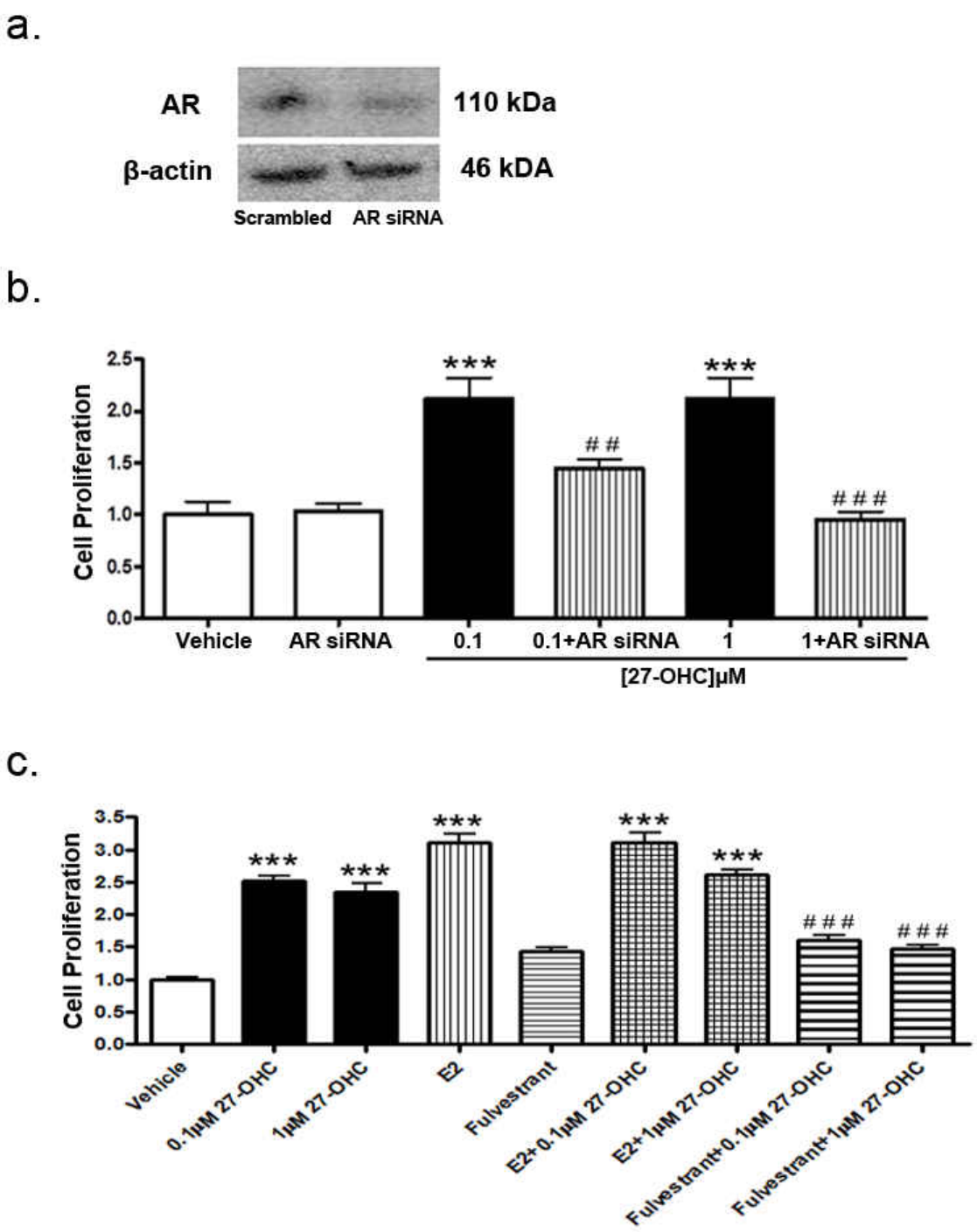


Figure 18: 27-OHC-induced cell proliferation is AR dependent. Western blot analysis (a) shows the efficiency of siRNA to AR with protein levels markedly

lower in RWPE-1cells treated with siRNA to AR than with scrambled siRNA. Cell proliferation assay (b) demonstrates that 27-OHC (0.1µM and 1.0 µM) increases cell proliferation and co-treatment with siRNA to AR markedly reduces proliferation. The assay was performed following a 24 hour incubation of RWPE-1cells with the siRNAs on a 96 well plate with their respective treatments. Cell proliferation assay (c) demonstrates that 27-OHC induced cell proliferation is attenuated when treated with 10µM fulvestrant. Readings were recorded 48 hours after the treatments. Data is expressed as Mean ± S.E.M. ***$p<0.001$ versus vehicle only, $^{\#\#}p<0.01$ versus 0.1µM 27-OHC only, $^{\#\#\#}p<0.001$ versus 0.1 µM or 1µM 27-OHC only treatment.

27-OHC suppressed docetaxel-induced apoptosis

Docetaxel is an apoptosis-inducing agent (Mhaidat et al. 2007) and the current chemotherapeutic drug of choice for advanced PCa (Petrylak 2000; Petrylak 2006; Hwang 2012; Kellokumpu-Lehtinen et al. 2013). To determine whether 27-OHC contributes to chemoresistance, we treated RWPE-1 cells with 27-OHC and docetaxel and monitored the number of apoptotic cells by TUNEL assay. We found that docetaxel induced apoptosis and 27-OHC (0.1 or 1.0 µM) markedly reduced docetaxel-induced apoptosis (Fig. 19). When an apoptotic signal is induced, caspase 3 (pro-enzyme) is activated by cleavage to execute apoptosis (Porter and Jänicke 1999; McIlwain et al. 2013). We found that docetaxel increased levels of both the pro and active caspase-3 (p17). Co-treatment with 27-OHC opposed the docetaxel-induced increase in pro caspase (Fig. 20 a,b) and active caspase-3 levels (Fig. 20 a,c).

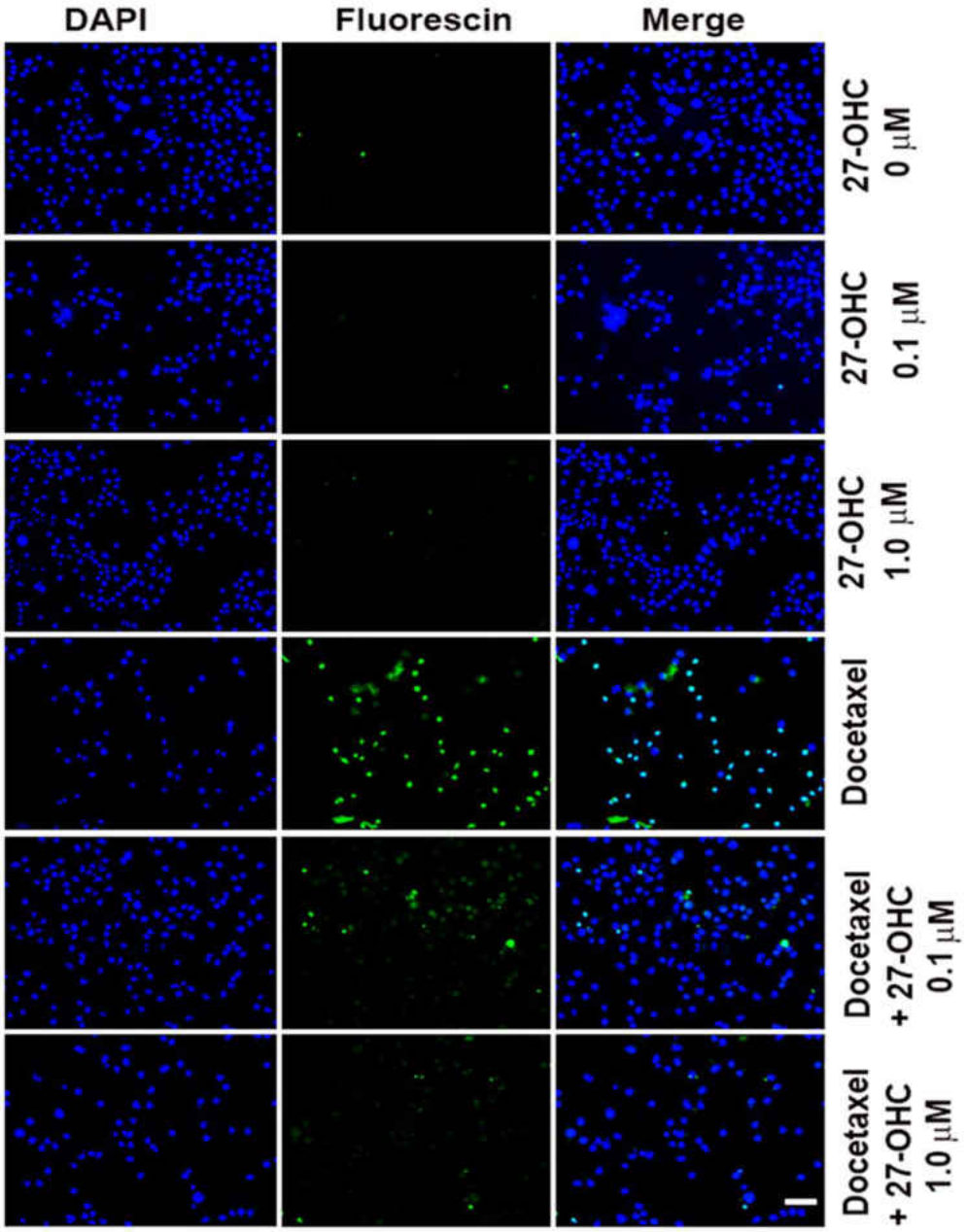
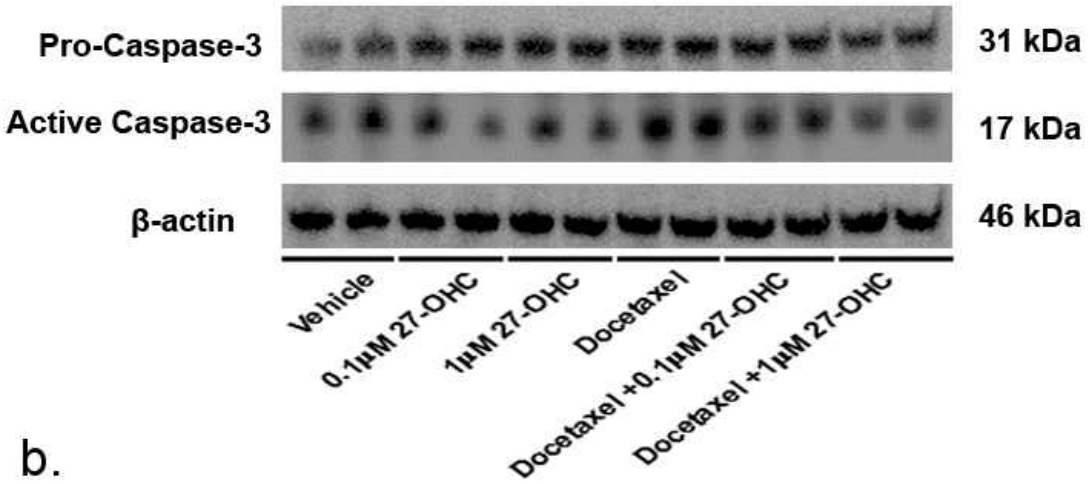


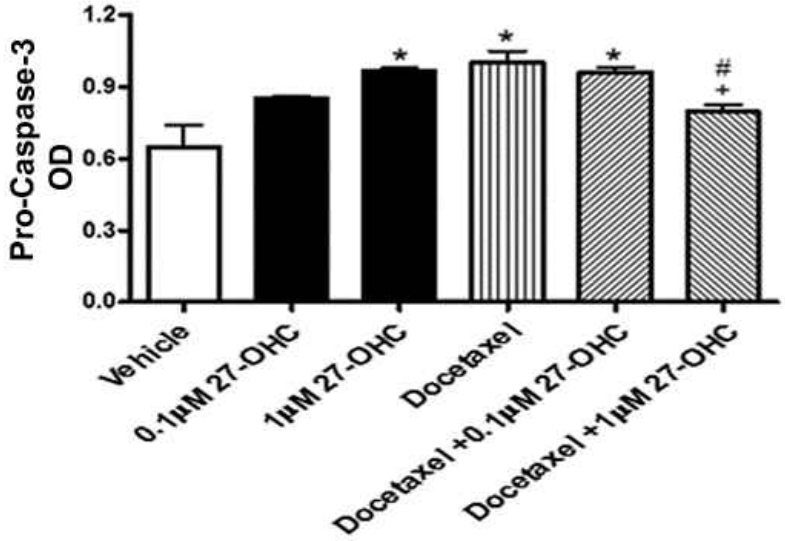
Figure 19: 27-OHC opposes docetaxel-induced apoptosis. Confocal images panels (a) and quantitation (b) for TUNEL assay in RWPE-1 cells treated with 27-OHC and/or docetaxel for 24 h. Docetaxel (0.1μM) induced a marked increase in

the apoptotic cells (Green) and treatment with 27-OHC at both 0.1 and 1.0 μM diminished apoptosis induced by docetaxel. Bar: 100μM.

a.


Pro-Caspase-3
31 kDa
Active Caspase-3
17 kDa
β-actin
46 kDa
Vehicle
0.1μM 27-OHC
1μM 27-OHC
Docetaxel
Docetaxel +0.1μM 27-OHC
Docetaxel +1μM 27-OHC

b.


Pro-Caspase-3 OD
1.2
0.9
0.6
0.3
0.0
*
*
*
#
+
Vehicle
0.1μM 27-OHC
1μM 27-OHC
Docetaxel
Docetaxel +0.1μM 27-OHC
Docetaxel +1μM 27-OHC

c.

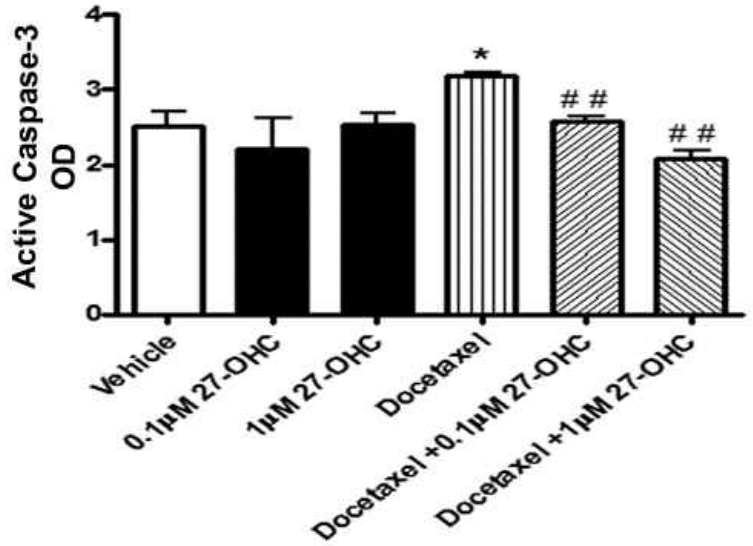

Active Caspase-3 OD
4
3
2
1
0
*
# #
# #
Vehicle
0.1μM 27-OHC
1μM 27-OHC
Docetaxel
Docetaxel +0.1μM 27-OHC
Docetaxel +1μM 27-OHC

Figure 20. 27-OHC reduces docetaxel-induced caspase-3 cleavage. Representative western blot (a) and densitometric analysis (b) showing a significant increase in pro-caspase protein levels in RWPE-1 cells when treated with 0.1µM docetaxel and concomitant treatment with 27-OHC markedly reduced pro-caspase protein levels. Representative western blot (a) and densitometric analysis (c) showing a significant increase in cleaved caspase (active, p17) protein levels in RWPE-1 cells when treated with 0.1µM docetaxel. Co-treatment with 27-OHC markedly reduced cleaved caspase protein levels. Data is expressed as Mean ± S.E.M. *$p<0.05$ versus controls, $^{\#}p<0.05$ versus docetaxel only treatment, $^{+}p<0.05$ and $^{++}p<0.01$ versus controls.

Discussion

This study was designed to determine the effects of 27-OHC on prostate cell proliferation and apoptosis. We also determined the involvement of AR in cell proliferation induced by 27-OHC. We demonstrate that 27-OHC increases proliferation and activates AR transcriptional activity in the normal prostate cells RWPE-1. We further demonstrate that 27-OHC-induced cell proliferation is AR dependent. Additionally, we show that 27-OHC opposes apoptotic cell death induced by docetaxel. Our data suggests that 27-OHC increases proliferation in normal epithelial prostate cells in an AR dependent manner.

27-OHC, the most abundant cholesterol metabolite in the circulatory system, is synthesized by the cytochrome P-450 enzyme, sterol 27 hydroxylase (CYP27A1), in the inner mitochondrial membrane of the liver and is metabolized by CYP7B1 enzyme (Fu et al. 2001; Ma et al. 2014 May 1). The main source of

27-OHC levels in the human body emanates from oxidation of cholesterol. As cholesterol levels increase following intake of diets rich in cholesterol or overproduction of endogenous cholesterol, production of 27-OHC increases. Patients with hypercholesterolemia were reported to have high levels of 27-OHC in their blood (Hirayama et al. 2009; Nelson et al. 2011; Nelson et al. 2013). Additionally, 27-OHC levels in the plasma have been shown to increase with age in males but not in females (Burkard et al. 2007a). Also, males are known to have higher basal levels of 27-OHC in plasma than females (Dzeletovic et al. 1995; Burkard et al. 2007a). 27-OHC is also known to activate or inhibit nuclear receptors depending on the target tissue. For instance, 27-OHC inhibits ER signaling in the vasculature (Umetani et al. 2007b) and bone (DuSell et al. 2010), however it activates ER signaling in breast (Cruz, Torres, María E. Ramírez, et al. 2010; Nelson et al. 2013; Wu et al. 2013).

To the best of our knowledge, there has been no established link between 27-OHC, PCa and BPH. In this study, we report an increase in cell proliferation in prostate cells in the presence of 27-OHC at concentrations below (0.1 µM) or above (1.0 µM) the physiological concentrations (Brown and Jessup 1999; Marwarha and Ghribi 2015). Total 27-OHC (esterified + non-esterified) is found in the human blood plasma ranging from 0.2 to 0.6 µM in a healthy individual (Hirayama et al. 2009; Schüle et al. 2010). Also, It has been shown that the levels of non-esterified 27-OHC, which is the most biologically active form of 27-OHC (Meaney et al. 2000; Meaney et al. 2001), is reported to be less than 20% of total 27-OHC in the human body (Burkard et al. 2007a; Ramirez et al. 2008;

Bandaru and Haughey 2014). In disease conditions, such as cancer and neurodegenerative diseases, macrophages migrate to affected areas and release excess 27-OHC in the surrounding tissue (Cruz et al. 2012; Nelson et al. 2013; Bandaru and Haughey 2014). We also found that fulvestrant, an ER inhibitor, attenuated 27-OHC induced cell proliferation to the same levels. Overall, our data suggests that 27-OHC may play an important role in prostate cell proliferation, which may result in the progression of BPH and/or PCa.

Androgen receptor (AR) plays an important role in prostate growth and development. There is a positive relationship between AR transcriptional activity and PCa progression (Heinlein and Chang 2004; Lonergan and Tindall 2011). Over 80% of PCa patients respond to anti-androgens or androgen deprivation therapy that targets and inhibits the AR activity (Heinlein and Chang 2004; Y. Chen et al. 2009). Additionally, androgens are also involved in BPH pathogenesis via AR (Lu et al. 2012; Izumi et al. 2013). However, the effects of cholesterol metabolites including the oxysterol 27-OHC on the AR signaling pathway remain to be determined.

The AR signaling pathway plays a critical role in the development and progression of PCa (Heinlein and Chang 2004; Lonergan and Tindall 2011; Izumi et al. 2013). AR signaling is chiefly targeted in the context of PCa using androgen ablation therapies (Heinlein and Chang 2004; Lonergan and Tindall 2011). As we found that AR transcriptional activity was increased with 27-OHC treatment, we determined the expression levels of prostate specific antigen (PSA), a well-known downstream target of AR, increased in these cells (Saxena et al. 2012).

PSA is a serine protease, which is produced by prostate epithelial cells and PCa cells. It is used as a serum biomarker to monitor PCa progression in patients (Kim and Coetzee 2004). Subsequently, we found that levels of PSA were increased. Also, using a ChIP assay, we demonstrate that binding of AR to the PSA promoter is increased in the presence of 27-OHC. We also show that 27-OHC-induced proliferation is AR-dependent. When AR gene expression was silenced, 27-OHC was unable to increase cell proliferation. As there was no additive effect in proliferation when cells were treated simultaneously with 27-OHC and E2 and because the ER selective inhibitor (Fulvestrant) prevented the 27-OHC-induced cell proliferation in RWPE-1 cells, we suggest the existence of an ER-AR crosstalk in the presence of 27-OHC since we show that the AR knockdown also reduces 27-OHC-induced proliferation.

Since 27-OHC levels increase with age (Burkard et al. 2007a), and 27-OHC induce AR transcriptional activity, 27-OHC may play a role in castrate-resistant prostate cancer. However, further studies are needed to evaluate the role of 27-OHC in castrate-resistant PCa.

Apoptosis is an important process that keeps the number of cells dividing under tight control (Hipfner and Cohen 2004). To assess the role of 27-OHC in regulating apoptosis we utilized a pro-apoptotic drug, docetaxel (Mhaidat et al. 2007), a current chemotherapeutic drug of choice for advanced PCa (Petrylak 2006; Kellokumpu-Lehtinen et al. 2013).To further understand whether 27-OHC plays a potential role in chemotherapeutic resistance, we treated cells with docetaxel and 27-OHC. We found that 27-OHC attenuated the pro-apoptotic

effects of docetaxel. While docetaxel is clinically used to battle metastatic PCa (McKeage and Keam 2005), it is also pro-apoptotic in non-tumorigenic RWPE-1 cells (Karanika et al. 2015). To better understand the role of 27-OHC in PCa further studies are needed, particularly metastatic PCa models. Our results warrant further investigation to understand the anti-apoptotic role of 27-OHC in chemotherapeutic resistance to PCa and BPH.

In summary, we demonstrate that 27-OHC induces an increase in proliferation in normal prostatic epithelial cells in an AR dependent manner. We also report for the first time, the docetaxel-resistant role of 27-OHC in epithelial cells. Our study provides a novel insight into the molecular mechanisms of 27-OHC and its role in modulating AR signaling pathway that is tightly linked to cell proliferation which is associated with PCa and BPH. Further studies are warranted to delineate the 27-OHC activated AR signaling pathway which may lead to unraveling novel therapeutic avenues for PCa and BPH.

CHAPTER IV

ROLE OF 27-HYDROXYCHOLESTEROL IN PROSTATE CANCER

The cholesterol metabolite, 27-hydroxycholesterol stimulates cell proliferation via ERβ in prostate cancer cells

Abstract

For every six men, one will be diagnosed with prostate cancer (PCa) in their lifetime. Estrogen receptors (ERs) are known to play a role in prostate carcinogenesis. However, it is unclear whether the estrogenic effects are mediated by estrogen receptor α (ERα) or estrogen receptor β (ERβ). Although it is speculated that ERα is associated with harmful effects on PCa, the role of ERβ in PCa is still ill-defined. The cholesterol oxidized metabolite 27-hydroxycholesterol (27-OHC) has been found to bind to ERs and act as a selective ER modulator (SERM). Increased 27-OHC levels are found in individuals with hypercholesterolemia, a condition that is suggested to be a risk factor for PCa. In the present study, we determined the extent to which 27-OHC causes deleterious effects in non-tumorigenic RWPE-1 cells, the low tumorigenic LNCaP cells, and highly tumorigenic PC3 prostate cancer cells. We found that incubation of LNCaP and PC3 cells with 27-OHC significantly increased cell proliferation. We also demonstrate that the ER inhibitor fulvestrant significantly reduced 27-OH-induced cell proliferation, indicating the involvement of ERs in

cell proliferation. Interestingly, ERβ levels, and to a lesser extent ERα, were significantly increased following incubation of PCa cells with 27-OHC. Furthermore, 27-OHC induced cell proliferation is attenuated in the presence of the ERβ specific inhibitor, PHTPP. Altogether, our results show for the first time that 27-OHC, through ER activation, triggers deleterious effect in prostate cancer cell lines. We propose that dysregulated levels of 27-OHC may trigger or exacerbate prostate cancer via acting on ERβ.

Introduction

Prostate cancer (PCa) is the second leading cause of death among men in the United States (Ferlay et al. 2010). The causes for PCa appear to be multifactorial, however it is well established that the incidence of PCa increases with age (Ferlay et al. 2010; Zhou et al. 2016). Several risk factors are associated with PCa including aging (Zhou et al. 2016), obesity (Gann 2002), hormonal imbalance (Gann 2002), oxidative stress (Gann 2002; Bostwick et al. 2004) and hypercholesterolemia (Magura et al. 2008; Moon et al. 2015).

ER signaling has been implicated in PCa; the isoform ERβ, and to a lesser extent ERα, is expressed in prostate epithelial and stromal cells (Bonkhoff et al. 1999; Hartman et al. 2012). ERα is considered pro-proliferative (Chakravarty et al. 2014) and ERβ anti-proliferative in the context of PCa (Weihua et al. 2002; Imamov et al. 2004; McPherson et al. 2008). However ERβ agonists have not shown clinical promise to combat PCa (Roehrborn et al. 2015) and there is a gap in knowledge elucidating the role of ERβ in PCa.

27-hydroxycholesterol (27-OHC) is the most abundant cholesterol metabolite in the periphery (Fu et al. 2001; Ma et al. 2014 May 1). Also, 27-OHC in plasma increases with age, especially in men (Burkard et al. 2007a). Men also have higher basal levels of 27-OHC in the plasma than women (Burkard et al. 2007a). Moreover, while patients with hypercholesterolemia are at risk for developing PCa (Magura et al. 2008), they also have increased 27-OHC levels in the blood (Hirayama et al. 2009; Nelson et al. 2011; Nelson et al. 2013). Understanding the role of 27-OHC in the context of PCa is critical and may reveal the underlying mechanisms responsible for PCa tumor initiation and progression. 27-OHC is a selective estrogen receptor modulator (SERM) that has been identified to bind to ER and modulate its activity (Umetani et al. 2007a; Umetani and Shaul 2011). 27-OHC levels are higher among those with hypercholesterolemia (Hirayama et al. 2009; Nelson et al. 2011; Nelson et al. 2013) and older men (Burkard et al. 2007b), both of which are at high risk of developing PCa. Also, 27-OHC-induced ER activation has been shown to promote ER+ breast cancer growth and progression (Cruz, Torres, María Eugenia Ramírez, et al. 2010; Nelson et al. 2013; Wu et al. 2013; Raza et al. 2015). Given that 27-OHC, at high levels, is associated with risk factors for PCa (i.e., hypercholesterolemia and aging) and that 27-OHC modulates ER signaling, a pathway that plays a role in PCa development and progression, it is essential to study the role of 27-OHC in the context of PCa. Further understanding of the role of 27-OHC in PCa may innovate alternative therapeutic avenues to those that are currently on the market.

We have previously shown that 27-OHC stimulates cell proliferation and inhibits docetaxel induced apoptosis in non-tumorigenic prostate epithelial cells (Raza et al. 2016). In this study, we determined a role for 27-OHC in activation of ERs, leading to deleterious effects in PCa cells.

Methods

Reagents

27-OHC was purchased from Santa Cruz Biotechnologies (Dallas, TX), docetaxel, 4-[2-Phenyl-5,7-bis(trifluoromethyl) pyrazolo[1,5-a]pyrimidin-3-yl]phenol (PHTPP) and Fulvestrant from Cayman Chemicals (Ann Arbor, MI) and β-estradiol from Sigma-Aldrich (St. Louis, MO). All cell culture reagents, with the exception of fetal bovine serum (FBS) (Atlanta Biologicals; Flowery Branch, GA) were from Invitrogen (Carlsbad, CA). Human RWPE-1, LNCaP and PC3 cells were purchased from ATCC (Manassas, VA).

Cell Culture

Non-tumorigenic human prostate epithelial RWPE-1 cells were maintained in Keratinocyte serum free medium (Invitrogen; Carlsbad, CA) supplemented with 0.05mg/ml BPE and 5ng/ml EGF. Metastatic LNCaP cells were maintained in RPMI 1640 medium and highly metastatic PC3 cells were maintained in F-12K medium. LNCaP and PC3 cells were supplemented with 10% FBS. All cells were supplemented with 100U/ml penicillin, 100µg/ml streptomycin 0.25µg/ml amphotericin (Sigma; St. Louis, MO) and cultured at 5% CO_2 and 37˚C. Stock solutions of 27-OHC were prepared in 100% ethanol and stored at -80°C. 27-

OHC stock solution was dissolved in appropriate volumes of media to prepare the working solutions of 1μM, a concentration that we showed to cause proliferation in prostate epithelial cells (Raza et al. 2016). Stock solutions of β-estradiol were dissolved in 100% ethanol and stored at -80°C. Stock solutions were diluted to prepare working solutions of 2nM. Stock solutions of PHTPP and fulvestrant were also dissolved in 100% ethanol, stored at -20°C, and diluted prior to treatment to prepare working solutions of 10μM. Concentrations of solvent in treatments were less than 0.1%. Our study was approved by the Institutional Biosafety Committee at the University of North Dakota.

Cell Proliferation Assay

Proliferation assays were conducted on black 96 well plates using CyQUANT Direct Cell Proliferation Assay (Invitrogen; Carlsbad, CA), which quantifies cell number using DNA content and membrane integrity. Cells seeded at 50-60% confluence were treated and incubated for 48 hours. Cells were then stained as per the manufacturer's protocol and read using Spectra MAX GEMINI EM (Molecular Devices; Sunnyvale, CA).

Metabolic Activity Assay (MTS assay)

Cell metabolic activity was quantified by measurement of the reduction of MTS to formazan product using CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega; Madison, WI) according to the manufacturer's protocol. The assay of the formation of formazan was performed by measuring absorbance

change using a microplate reader (Molecular Devices; Sunnyvale, CA) 48 h after treatments.

Western Blot Analysis

Treated cells were washed with PBS, trypsinized, and centrifuged at 5000 g. The pellets were washed with PBS and homogenized in M-PER tissue protein extraction reagent (Thermo Scientific; Waltham, MA) supplemented with protease and phosphatase inhibitors. Denatured proteins (5 µg) were separated in 10% SDS-PAGE gels, transferred to a PVDF membrane (Millipore; Billerica, MA) and incubated with antibodies to ERα (1:1000, Santa Cruz; Dallas,TX) and ERβ (1:1000, Millipore, Billerica, MA). β-actin was used as a gel loading control. The blots were developed with enhanced chemiluminescence (ECL Clarity kit, Bio-Rad). Bands were visualized on a polyvinylidene difluoride membrane and analyzed by LabWorks 4.5 software on a UVP Bioimaging System. Quantification of results was performed by densitometry and the results analyzed as total integrated densitometric values (arbitrary units).

Invasion assay

Invasion Assays were conducted using QCM 96-well Cell Invasion Assay kit (Millipore; Billerica, MA). The various treatments were added to the wells of the feeder tray and at least $1x10^4$ cells/well re-suspended in serum free medium were added to the invasion chambers. Cells were incubated with treatments for 24 hours then stained as per manufacturer's protocol and transferred to a black

96 well plate to read fluorescence using Spectra MAX GEMINI EM (Molecular Devices; Sunnyvale, CA).

Real-time Polymerase Chain Reaction (RT-PCR)

After treatments, cells were lysed according to QuickGene Mini80 protocol and kit (Autogen). RNA sample were quantitated by spectrophotometry and subsequently, 1µg total RNA was used as template to synthesize cDNA with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems; Foster City, CA). We selected genes involved in pathways related to 27-OHC, including cholesterol metabolism (Björkhem 2013), liver X receptor (LXR) (Lehmann et al. 1997) and sonic hedgehog (Shh) (Javitt 2008) signaling. We also selected genes involved in metastasis (Gorlov et al. 2010; Wang et al. 2013; Broustas and Lieberman 2014), oxysterol binding (Vesa M. Olkkonen et al. 2012) and tumor suppression(Bereczki et al. 2008). The screened genes are included in Table I. Primers for all assays were designed using Primer Express 3.0 (Applied Biosystems; Foster City, CA). Melting curve analysis was performed to ensure single-product amplification for all primer pairs. Real time PCR was performed on the ABI 7900HT Fast Real Time PCR System (Applied Biosystems; Foster City, CA) using the panel of genes of interest. Data analysis was performed using Sequence Detection System software from Applied Biosystems, version 2.4. The experimental Ct (cycle threshold) was calibrated against the endogenous control products alpha-ACTIN (ACTN1) and Beta-2-Microglobulin (B2M). Samples were analyzed for relative gene expression by the DDCt method (Pfaffl 2001).

Table 1: Selected Genes analyzed with their corresponding pathways

Cholesterol Metabolism			Nuclear receptor signaling			Shh signaling		
CYP27A1	CYP7B1	HMGCR	ABCA1	EZH2	FOXA1	GLI1	GLI2	GLI3
NFKB1	SRD5A2	SREBF1	NR1H2	NR1H3	RXRA	PTCH1	PTCH2	SHH
			RXRB	SIRT1	TFF1	SMO		
			TMPRSS2					

Metastasis			Oxysterol Binding		Tumor Suppression		
CBX1	CBX5	CTGF	INSIG2	OSBP	MDM2	TAF3	TNF
FN1	IGF-1	IGFBP3			TP53		
MTA1	MTA3	SNAI1					
SNAI2	SPARC	VEGFA					

Table 2: Primers used in determining mRNA expression of significantly regulated genes

Gene	Sequence
CBX1-f	TGAGCAGCGTCACCCTTTACAC
CBX1-r	CCACTTTGCCCTTTACCACTCG
CBX5-f	TGGCACAATCTTGGCTTACTGT
CBX5-r	ATGGTGGCACACACCTGTAGTC
CTGF-f	AGGATGTGCATTCTCCAGCCATC
CTGF-r	TGTCAGAGCTGAGTCTGCTGTTC
CYP27A1-f	CAGCTGCGCTTCTTCTTTCAGC
CYP27A1-r	TGGCCTTGTAAAGCACCTGTAAC
FOXA1-f	TCCTCAGGAATTGCCCTCAAGAAC
FOXA1-r	ATGACATGACCATGGCACTCTGC
GLI2-f	TGTCTGAGTGACACCAACCAGAAC
GLI2-r	TGTGAATGGCGACAGGGTTGAC
GLI3-f	CCTCCAGCACCACTTCTAATGAGG
GLI3-r	TCTGTGGCTGCATAGTGATTGCG
IGFBP3-f	TCCAAGCGGGAGACAGAATATGG
IGFBP3-r	AGGAACTTCAGGTGATTCAGTGTG
INSIG2-f	CATGCCAGTGCTAAAGTGGATTTC
INSIG2-r	TGGATAGTGCAGCCAGTGTGAG
MDM2-f	TCCTCTCAAGCTCCGTGTTTG
MDM2-r	TCATGATGTGGTCAGGGTAGATG
MTA3-f	GCAGCAGAAGCTGAGAGTAAACTG
MTA3-r	TGGTTGGGATTTGGTTTGCTGTAG
NR1H2-f	GCATCCACTATCGAGATCATGCTG
NR1H2-r	GAAGGTGATACACTCTGTCTCGTG
OSBP-f	CTATGAAAGCCACAGAGGATGGC
OSBP-r	GTCCTTCTTCCGCTCAAACCAC
PTCH1-f	TCACCGTTCACGTTGCTTTGGC
PTCH1-r	AAACATGTGCTCCAGGGCAAGC

Gene	Sequence
RXRB-f	TGCTGTGGAACAGAAGAGTGACC
RXRB-r	ATGTTAGTCACAGGGTCATTTGGG
SIRT1-f	ACAGGTTGCGGGAATCCAAAGG
SIRT1-r	CCTAGGACATCGAGGAACTACCTG
SPARC-f	TGGCGAGTTTGAGAAGGTGTGC
SPARC-r	TGGCAAAGAAGTGGCAGGAAGAG
SRD5A2-f	ACATACGGTTTAGCTTGGGTGTC
SRD5A2-r	TTTCTCCAGGCTTCCTGAGCTG
SREBF1-f	GCCATGGATTGCACTTTCGAAGAC
SREBF1-r	GGTCAAATAGGCCAGGGAAGTCAC
TAF3-f	GCCATCGGTACTCTGAGCTCTATG
TAF3-r	TGACGGAATTTGGTGTGGGAAGG
TFF1-f	GCCCCCCGTGAAAGACA
TFF1-r	CGTCGAAACAGCAGCCCTTA
TMPRSS2-f	TGTGGTCCCTTCCAATGCTGTG
TMPRSS2-r	TGCTCATGGTTATGGCACTTGGC
TNF-f	CCAGGCAGTCAGATCATCTTCTCG
TNF-r	ATCTCTCAGCTCCACGCCATTG
TP53-f	AGTGGAAGGAAATTTGCGTGTGG
TP53-r	TGGTACAGTCAGAGCCAACCTC
VEGFA-f	AGGGCAGAATCATCACGAAGTGG
VEGFA-r	AGGGTCTCGATTGGATGGCAGTAG
ACTINalpha-f	CAGGACCGTGTGGAGCAGATTG
ACTINalpha-r	CAGATTGTCCCACTGGTCACAG
B2M-f	TGAGTATGCCTGCCGTGTGAAC
B2M-r	TGCTGCTTACATGTCTCGATCCC

Immunocytochemistry (ICC)

Coverslip seeded cells were rinsed with PBS and fixed in cold acetone, blocked with 10% normal goat serum and incubated overnight at 4˚C with human anti-ERβ2 monoclonal antibody (Biorad; Hercules, CA). ERβ2 was conjugated to Alexa Fluor 488. All coverslips were washed and mounted with Vectashield containing DAPI. Slides were visualized using DMI 6000 (Leica Microsystems; Buffalo Grove, IL).

Statistical analysis

Differences among groups were assessed by unpaired t-test and One-Way Analysis of Variance (One-Way ANOVA) followed by Tukey's post-hoc test. Statistical analysis was performed with GraphPad Prism software 4.01. Quantitative data for experimental analysis are presented as mean values ± S.E.M with unit value assigned to control and the magnitude of differences among the samples being expressed relative to the unit value of control.

Results

The cholesterol metabolite 27-OHC increases cell proliferation in PCa cells

We have previously shown that 27-OHC stimulates cell proliferation in non-tumorigenic RWPE-1 cells (Raza et al. 2016). However, the effects of 27-OHC on proliferation in PCa cells were not determined. Here we show that 27-OHC stimulates cell proliferation in PCa cells, LNCaP and PC3. Upon 27-OHC treatment, cell proliferation was increased by ~60% in LNCaP and ~30% in PC3

compared to their respective controls (Fig 21a,b). To confirm our results, we performed MTS assay which measures mitochondrial activity of the cells. We found that 27-OHC also significantly increases metabolic activity of the both cells (Fig 21c,d). These results suggest that 27-OHC induces cell proliferation in PCa cells.

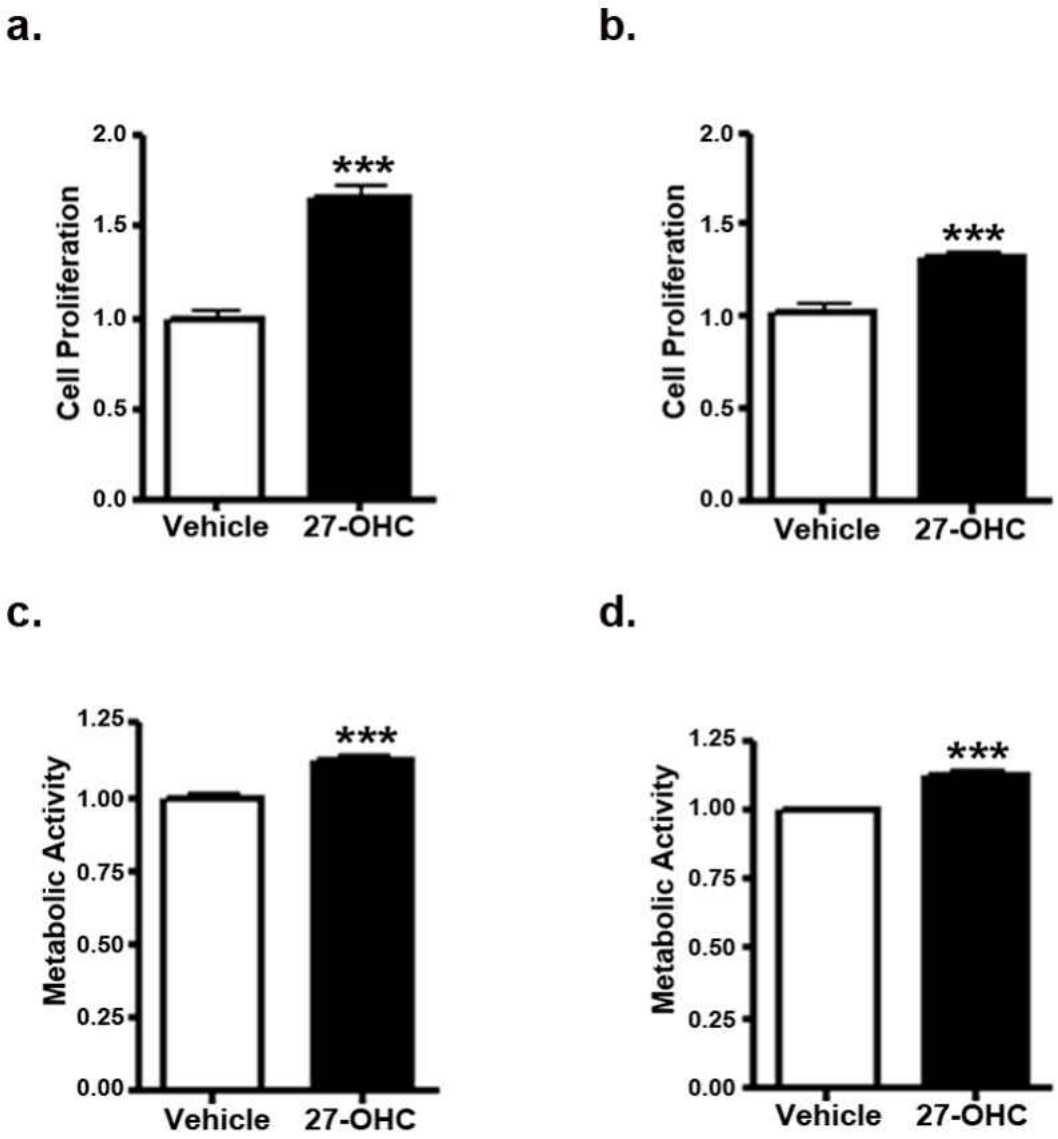


Figure 21: 27-OHC induces cell proliferation in PCa cells. Cell proliferation assay in LNCaP (a) and PC3 (b) cells demonstrates a significant increase in proliferation in the presence of 27-OHC. MTS assay shows a significant increase in cell metabolic activity in the presence of 27-OHC in LNCaP (c) and PC3 (d) cells. Cells were treated with 1µM 27-OHC. Readings were recorded 48 hours

after treatment with 27-OHC. Data is expressed as Mean ± S.E.M. ***p<0.001 versus controls.

27-OHC stimulates cell proliferation via ER

Since 27-OHC is a ligand of ER (Umetani and Shaul 2011) and that 27-OHC-induced ER modulation leads to increased cell proliferation in breast cancer cells (Cruz, Torres, María E. Ramírez, et al. 2010; Nelson et al. 2013; Wu et al. 2013; Raza et al. 2015), we assessed the importance of ERs in 27-OHC-induced cell proliferation in PCa cells. We have previously shown that 27-OHC induced cell proliferation in non-tumorigenic prostate epithelial cells was ER dependent (Raza et al. 2016). Here, we show that the ER specific inhibitor ICI 182,780 (fulvestrant) (McKeage et al. 2004) mitigated 27-OHC induced cell proliferation to basal levels in LNCaP and PC3 cells (Fig 22. a,b). Also, we found that upon concomitant treatment of 27-OHC and estradiol (E2), the natural agonist of ER (Hall et al. 2001), there was no additive effect in cell proliferation in both cells (Fig 22. a,b). These results suggest that ER activation is necessary for 27-OHC induced cell proliferation.

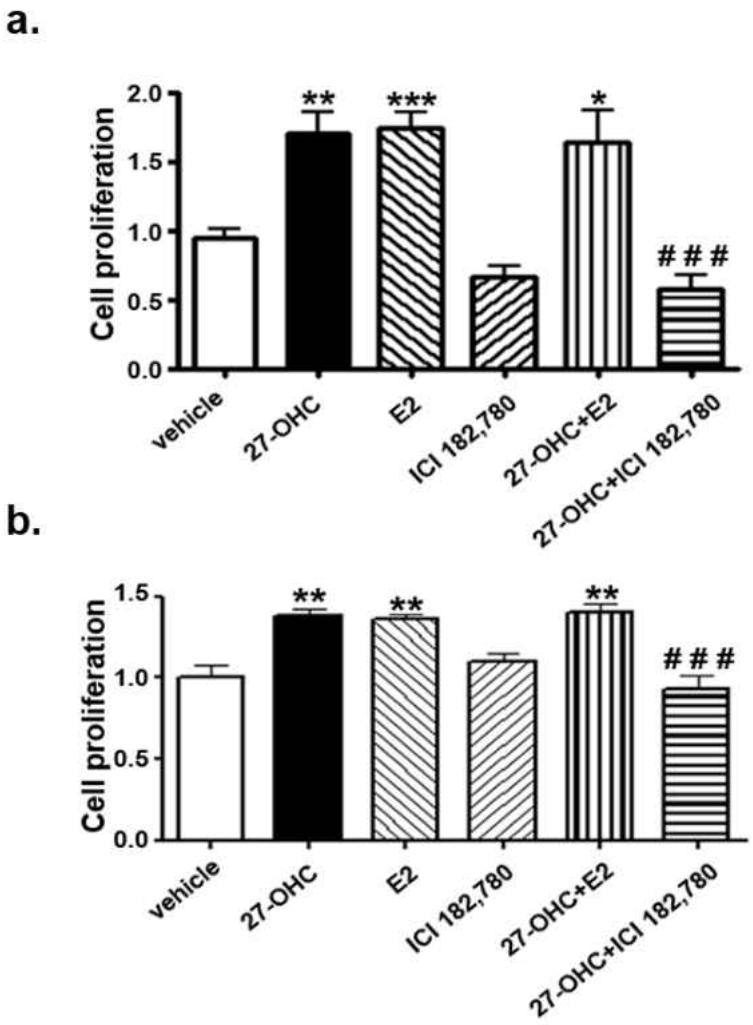


Figure 22: 27-OHC stimulates cell proliferation via ER. Cell proliferation assay in LNCaP (a) and PC3 (b) cells demonstrates an attenuation of 27-OHC-induced cell proliferation with the ER inhibitor ICI 182,780 (Fulvestrant). Cells were treated with 1µM 27-OHC, 2nM of E2 and 10µM ICI 182,780. Readings were recorded 48 hours after treatment with 27-OHC. Data is expressed as Mean ± S.E.M. **p<0.01; ***p<0.001versus controls, ###p<0.001 versus 27-OHC only treatment.

27-OHC selectively up-regulates ERβ expression

Given that 27-OHC stimulates cell proliferation in non-tumorigenic (Raza et al. 2016) as well as PCa cells (Fig. 21a,b) and that 27-OHC is a ligand of both isoforms of ER, ERα and ERβ (DuSell et al. 2010; Umetani and Shaul 2011), we determined the extent to which 27-OHC regulates ERα and ERβ protein levels. We found that 27-OHC had no significant effects on ERα levels, but significantly up-regulated ERβ levels in the non-tumorigenic RWPE-1 (Fig. 23a-c) and PCa cells (Fig. 23d-i). When compared to vehicle treated, 27-OHC treated cells exhibit an increase in ERβ levels by ~250% in RWPE-1 (Fig. 23c), ~100% in LNCaP (Fig. 23f), and ~50% in PC3 (Fig. 23i). This data suggests a potential involvement of ERβ in 27-OHC-induced cell proliferation.

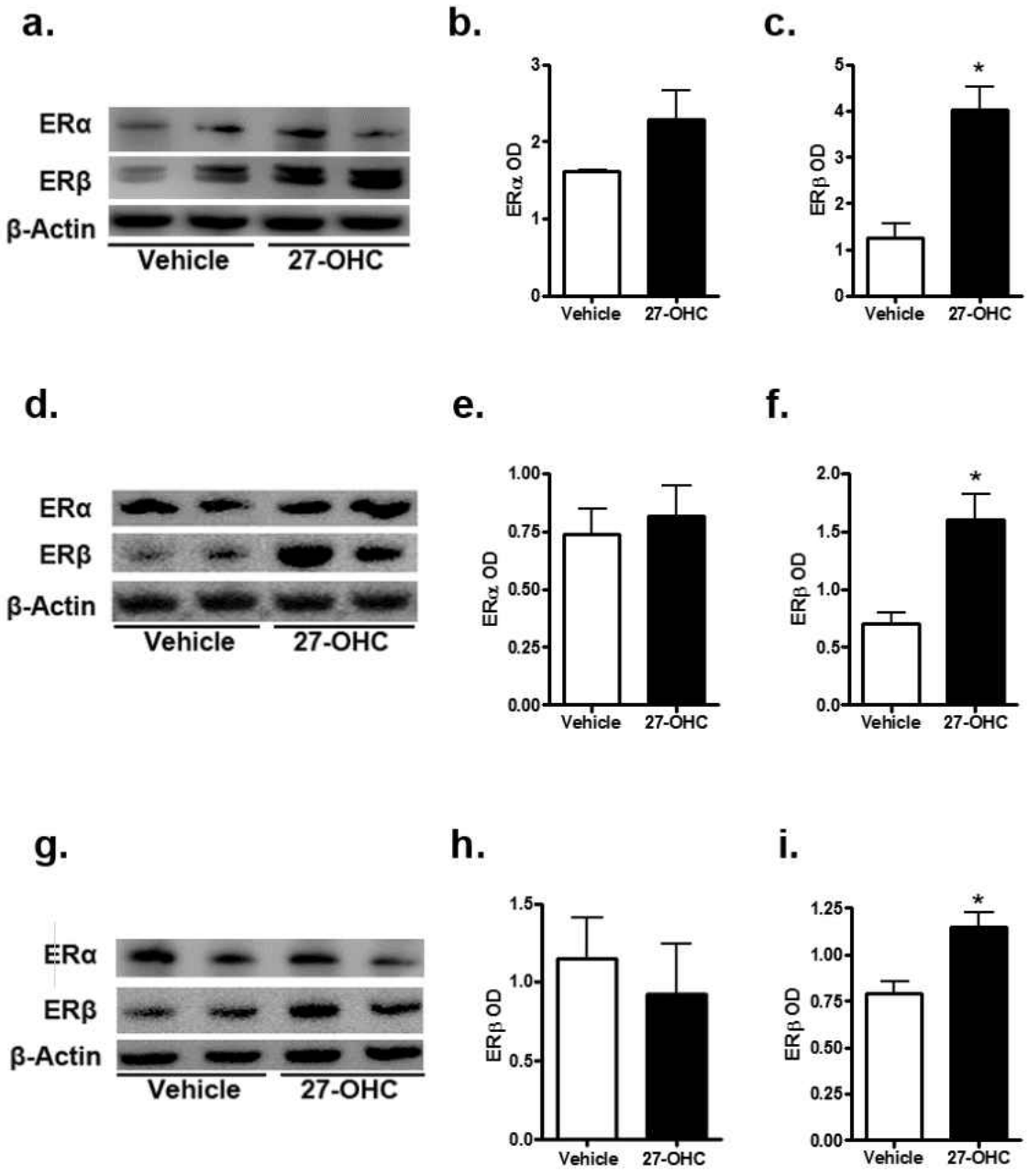


Figure 23: 27-OHC upregulates ERβ expression. Representative western blots (a) and densitometric analysis showing no significant change in ERα expression in RWPE-1 (b) and a significant increase in ERβ expression (c) in RWPE-1 cells. Representative western blots (d) and densitometric analysis showing no

significant change in ERα expression (e) and a significant increase in ERβ expression in LNCaP cells. Representative western blots (g) and densitometric analysis showing no significant change in ERα expression (h) and a significant increase in ERβ expression in PC3 cells (i). Data is expressed as Mean ± S.E.M. *$p<0.05$ versus controls.

27-OHC induces cell proliferation via ERβ

To determine if 27-OHC-induced cell proliferation is due to ERβ specific activation, we utilized an ERβ specific antagonist PHTPP (Compton et al. 2004). Upon treatment of the non-tumorigenic and PCa cells with 27-OHC and PHTPP, PHTPP attenuated 27-OHC-induced cell proliferation to basal levels in all cells (Fig. 24a-c). This data suggests that ERβ activation is essential for 27-OHC-induced cell proliferation.

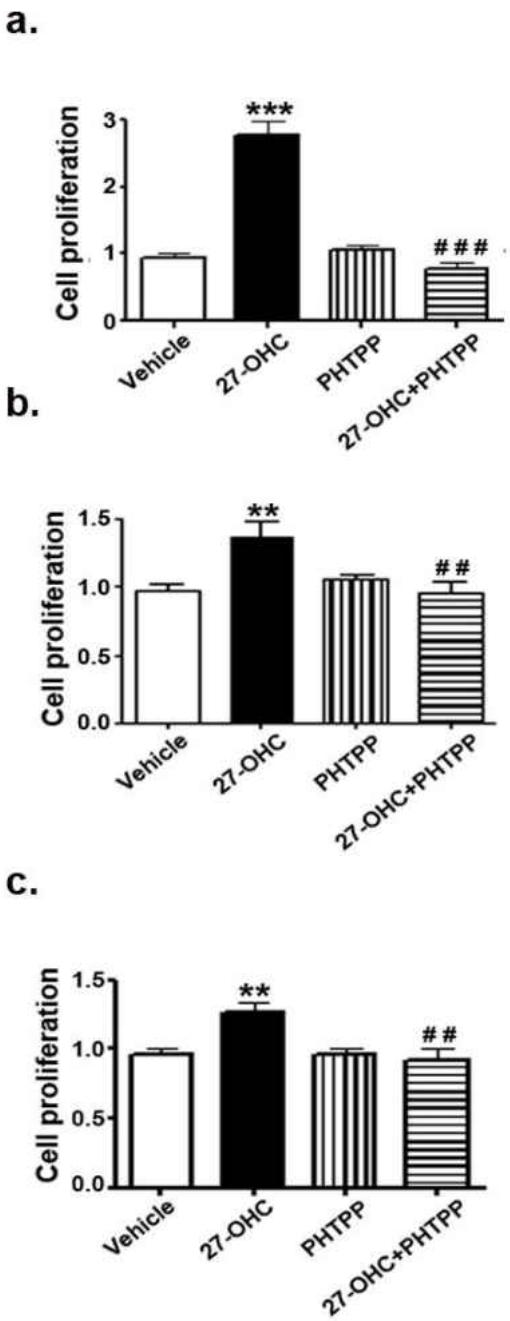


Figure 24: 27-OHC induces cell proliferation via ERβ. Cell proliferation assay in RWPE-1 (a), LNCaP (b) and PC3 (c) cells demonstrates attenuation of 27-OHC-induced cell proliferation with PHTPP treatment. Cells were treated with 1μM 27-OHC and 10μM PHTPP. Readings were recorded 48 hours after treatments.

Data is expressed as Mean ± S.E.M. **p<0.01; ***p<0.001 versus controls, ##p<0.01; ###p<0.001 versus 27-OHC only treatment.

27-OHC reduces cell invasion in PCa but not in non-tumorigenic cells

Cell invasion is a key process by which cancerous cells further tumor progression and metastasize to distant tissues and organs. Metastatic cells invade healthy tissue by penetrating through the extracellular matrix (ECM) of healthy cells (Krakhmal et al. 2015). To investigate the effect of 27-OHC on cell invasion, we treated cells with 27-OHC and determined the change in cell invasion across the ECM. We found that upon 27-OHC treatment, cell invasion did not significantly change in the RWPE-1 cells (Fig. 25a) but significantly decreased in LNCaP and PC3 cells (Fig. 25b,c). Also, upon PHTPP treatment only, cell invasion significant decreased in non-tumorigenic RWPE-1 and LNCaP cells but not in PC3 cells (Fig. 25a-c). Interestingly, PHTPP rescued 27-OHC-induced decrease in cell invasion of PC3 cells (Fig. 25c). This data suggests that 27-OHC has no effect on normal prostate but inhibits cell invasion in PCa cells.

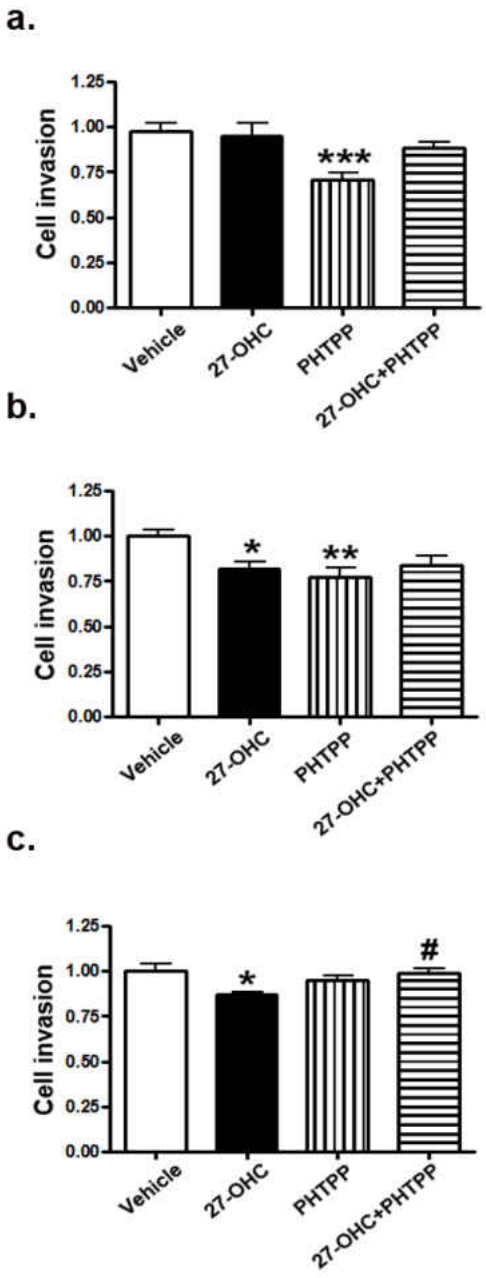


Figure 25: 27-OHC reduces ECM invasion in PCa cells but not in non-tumorigenic cells. Cell invasion assay demonstrates that while there was no change in invasion in RWPE-1 cells treated with 27-OHC (a), a significant decrease in cell invasion occurred in LNCaP (b) and PC3 (c) cells treated with 27-OHC. Cells were treated with 1μM 27-OHC and 10μM PHTPP. Readings

were recorded 48 hours after treatment with 27-OHC. Data is expressed as Mean ± S.E.M. *p<0.05, **p<0.01, ***p<0.001 versus controls, #p<0.05 versus 27-OHC only treatment.

27-OHC differentially regulates gene expression in non-tumorigenic and PCa cells.

We determined the extent to which 27-OHC related gene expression is regulated in normal prostate epithelial RWPE-1 cells and PCa cells (Table 3). We found that in non-tumorigenic RWPE-1 cells, the expression levels of TFF1 (PS2) and TMPRSS2 were significantly upregulated by 27-OHC treatment. In LNCaP cells, CTGF, IGFBP-3, INSIG2, NR1H2 and RXRB was significantly upregulated while expression of SREBF-1 and TMPRSS2 was significantly down-regulated. In PC3 cells, expression of CBX1, CBX5, CYP27A1, CTGF, FOXA1, GLI2, GLI3, MDM2, MTA3, OSBP, PTCH1, RXRB and SIRT1 was significantly upregulated and only SPARC expression was significantly downregulated (Table 3). The sequences for primers of the significantly regulated genes are presented in Table 2. This data emphasizes the inherent differences in the cells and the potential genes regulated by 27-OHC in the different prostate cell lines.

Table 3: Significantly regulated genes in fold change

Genes	RWPE-1	LNCaP	PC3
CBX1	0.9956	1.0503	1.2222*
CBX5	1.1252	1.0153	1.2895**
CTGF	0.8859	3.5324*	1.4869**
CYP27A1	0.5065	N/E	1.2263**
FOXA1	1.1357	1.1356	1.1534**
GLI2	0.9029	0.8980	1.3012***
GLI3	0.9287	0.9672	1.1714*
IGFBP3	0.9773	2.3619*	1.1511
INSIG2	0.9938	1.1923*	1.1851
MDM2	1.0007	1.0855	1.2051*
MTA3	1.0713	1.1684	1.2135**
NR1H2	0.9437	1.1690*	1.1723
OSBP	1.1333	1.1635	1.2088*
PTCH1	0.9376	1.3609	1.3304*
RXRB	1.1306	1.2035*	1.2004**
SREBF1	0.6093	0.6811*	1.1461
SIRT1	0.9817	1.1805	1.2671*
SPARC	0.9115	1.0014	0.8155***
TFF1	1.8358*	1.3250	1.0190
TMPRSS2	1.4082*	0.8474*	1.1262

*N/E denotes not expressed.*p<0.05, **p<0.01, ***p<0.001 versus controls*

27-OHC regulates ERβ2 expression and cellular localization

ERβ is categorized into several isoforms including ERβ1, ERβ2, ERβ4 and ERβ5 which are expressed in the prostate gland (Christoforou et al. 2014) . ERβ1 is the only functional isoform with a ligand binding domain while the activity of the other isoforms may depend on ERβ1 expression and isoform ratios (Christoforou et al. 2014). The ERβ1 isoform is the most studied isoform which is known to have a protective role in prostate cancer while ERβ2 is considered deleterious (M. Chen et al. 2009; Nelson et al. 2014) and known to correlate with poor prognosis (Leung et al. 2010). To test whether 27-OHC regulates ERβ2 expression in prostate cells, we treated cells with 27-OHC and stained for ERβ2. We found that while ERβ2 is expressed in a punctated fashion in the nucleus and the cytoplasm in RWPE-1 cells, 27-OHC appeared to increase overall ERβ2 expression (Fig. 26a). In LNCaP cells, we saw no changes in ERβ2 staining intensity (Fig. 26b.) and in PC3 cells we found that 27-OHC appeared to have no overall effect on ERβ2 expression (Fig. 26c), however interestingly, stained ERβ2 punctates in the nucleus decreased when treated with 27-OHC. This data suggests that 27-OHC alters the ERβ2 expression and cellular localization depending on the prostate cell line.

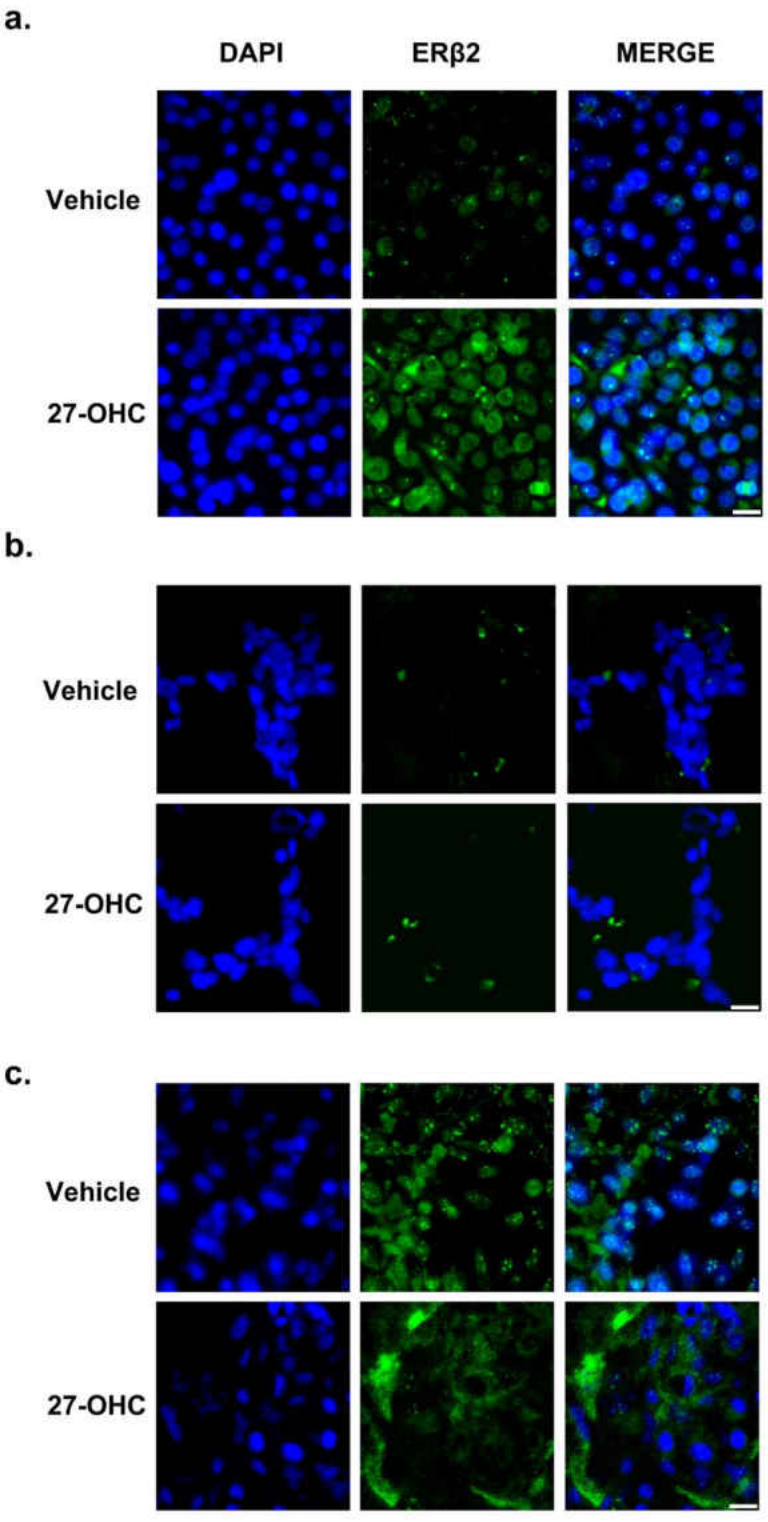

a.
DAPI
ERβ2
MERGE
Vehicle
27-OHC
b.
Vehicle
27-OHC
c.
Vehicle
27-OHC

Figure 26: 27-OHC differentially regulates ERβ2. Representative fluorescence microscopy images of prostate cells depicting ERβ2 (green) expression & localization and the nucleus (blue). (a) RWPE-1 cells treated with 27-OHC showed increased intensity of ERβ2. (b) LNCaP cells treated with 27-OHC depicted no change in ERβ2 staining intensity and (c) PC3 cells treated with 27-OHC demonstrated no change in overall ERβ2 staining intensity, but a decrease in nuclear ERβ2. All cells were treated with1 μM 27-OHC for 24 hr. Bar, 50μm.

Discussion

This study investigated the role of 27-OHC in PCa cell models. We demonstrate that 27-OHC induces cell proliferation in PCa cells through ER. We further show that 27-OHC regulates ERβ expression over ERα. Moreover, we demonstrate that 27-OHC-induced cell proliferation is dependent on ERβ activation. We also show that 27-OHC reduces ECM cell invasion in PCa cells but not in non-tumorigenic cells. Additionally, we found that several genes related to oxysterol biology were differentially expressed following 27-OHC treatment in non-tumorigenic and PCa cells. Thus, our data show for the first time a potential link between 27-OHC and PCa pathogenesis by demonstrating the deleterious effect of 27-OHC in PCa cellular models.

Previously, we have reported that 27-OHC stimulates cell proliferation in non-tumorigenic prostate epithelial cells (Raza et al. 2016). Here, we demonstrate the effect of 27-OHC in PCa cells. Given that cell proliferation is associated with tumor growth, we measured cell proliferation upon 27-OHC treatment. We found that 27-OHC significantly increases cell proliferation in

tumorigenic LNCaP and PC3 cells. Interestingly, the magnitude of 27-OHC-induced cell proliferation in LNCaP is higher than PC3. Also, the magnitude of 27-OHC-induced cell proliferation is higher in RWPE-1 than in LNCaP and PC3. The three epithelial cell lines have different morphologies, androgen receptor (AR) status, and depict different stages of PCa pathology. RWPE-1 are non-tumorigenic (Bello et al. 1997), LNCaP are androgen sensitive and with low tumorigenicity (Sato et al. 1997), and PC3 are androgen insensitive and highly tumorigenic (Kaighn et al. 1979). Taking tumorigenicity of the cells into account, it appears that 27-OHC increases cell proliferation to a greater magnitude in prostate cells with low or non-tumorigenic phenotypes versus highly tumorigenic phenotype. This may suggest the potential role of 27-OHC in early stages of PCa. Further studies are warranted to study the role of 27-OHC in forming PCa tumors.

The role of estrogens and estrogen receptors in the context of PCa is currently being explored. Recently, estrogens have been associated with the development and progression of PCa (Nelles et al. 2011). Moreover, the discovery of 27-OHC as a SERM (Umetani and Shaul 2011) adds another dimension to the growing phenomena of estrogenic actions as well as cholesterol metabolism role in PCas. Our previous (Raza et al. 2016) and current data strongly suggest that 27-OHC-induced cell proliferation in non-tumorigenic prostate cells and in PCa cells is ER dependent. When cells were treated concomitantly with 27-OHC and the ER inhibitor fulvestrant, proliferation was substantially attenuated. Given that 27-OHC is known to bind and activate ER

(Umetani and Shaul 2011; Wu et al. 2013), our results suggest that activation of ER is required to ensue 27-OHC-induced cell proliferation in PCa cells. This observation suggests a potential link between 27-OHC, ER and PCa.

It is worth noting that 27-OHC can act as an ER agonist or ER antagonist depending on the target tissue. For example, 27-OHC activates ER in breast tissue (Cruz, Torres, María E. Ramírez, et al. 2010; Nelson et al. 2013; Wu et al. 2013) and inhibits the receptor in the vasculature (Umetani et al. 2007b). We propose an additional novel concept that 27-OHC activates ER signaling in prostate cells and may thus play a pivotal role in PCa development and progression.

Furthermore, we demonstrate that 27-OHC-induced cell proliferation in non-tumorigenic and PCa cells is ERβ dependent. When cells are treated with the ERβ specific inhibitor PHTPP, 27-OHC-induced cell proliferation is attenuated, suggesting the 27-OHC-induced ERβ activation as the underlying factor leading to cell proliferation.

Our results demonstrate that 27-OHC reduces cell invasion in PCa cells but not in non-tumorigenic cells. The finding that 27-OHC increases cell proliferation but reduces cell invasion is unexpected, however it is in accordance with the current environment of diagnosed PCa in which over 90% of PCa diagnosed are reported to be localized PCas[51,52]. Additionally, it is important to note that cell invasion and proliferation are two different parameters in cancer. Moreover, androgen deprivation therapy(ADT) for patients with localized PCa does not improve survival or delay the use of secondary cancer therapy[53],

suggesting a potential role of 27-OHC-induced estrogenic signaling in localized PCa.

Our study measured expressions of genes regulated by 27-OHC in non-tumorigenic cells and PCa cells. In non-tumorigenic RWPE-1 cells, we found that 27-OHC treatment upregulated TFF1 (ps2) and TMPRSS2, downstream targets of ER (Shao et al.) and AR (Lucas et al. 2008), respectively. The upregulation of these target genes by 27-OHC demonstrates that this oxysterol activates both ER and AR target genes. This corroborates our earlier report that AR and ER are necessary to induce cell proliferation in 27-OHC-treated RWPE-1 cells (Raza et al. 2016). This observation also substantiates the idea that ER and AR activation simultaneously play a significant role in PCa tumor initiation. For instance, when Noble rats are concomitantly treated with estradiol and testosterone almost all rats develop PCa tumors, while only 40% of them develop PCa when treated with testosterone alone (Bosland et al. 1995; Bosland 2005).

Unlike in RWPE-1 cells, we did not find upregulation of ps2 and TMPRSS2 genes by 27-OHC in PCa cells. This may be attributed to the fact that LNCaP has a mutated AR (Veldscholte et al. 1992) and that PC3 does not express AR (Tai et al. 2011). In PCa cells, we found that the connective tissue growth actor (CTGF), which plays a vital role in tumorigenesis and wound healing processes (Jacobson and Cunningham 2012) is upregulated by 27-OHC. Also, specific to LNCaP, we found a significant upregulation of insulin-like growth factor binding protein-3 (IGFBP-3) which has been implicated in PCa tumors.

IGFBP-3 is an anti-angiogenic and anti-metastatic protein that is upregulated and localized in the nucleus of PCa tumor cells (Seligson et al. 2013).

Moreover, our results demonstrate that ERβ2 can be regulated by 27-OHC. In RWPE-1, the cells which obtained the most increase in cell proliferation upon 27-OHC treatment, ERβ2 expression increased. Surprisingly while no change was observed in LNCaP, PC3 demonstrated a reduction in nuclear ERβ2 expression, which corresponds to the decreased cell invasion upon 27-OHC treatment (Fig 5c). This finding corroborates with the observation that nuclear ERβ2 abundance is associated with poor PCa prognosis and increased cell invasion(Leung et al. 2010).

Given the following observations; 27-OHC induced AR transactivation and increased cell proliferation in an AR dependent manner in RWPE-1 cells & 27-OHC does not directly bind to AR (Raza et al. 2016), RWPE-1 is the only cell line in this study with a wild type AR (Bello et al. 1997) and it is also the only cell line to show an increase in ERβ2 expression upon 27-OHC treatment. Furthermore, 27-OHC augmented cell proliferation at a greater magnitude in non-tumorigenic (RWPE-1) compared to low tumorigenic (LNCaP) and highly tumorigenic PCa cells (PC3). Taken together, we hypothesize that 27-OHC may bind and activate ERβ inducing downstream AR-ERβ2 crosstalk signaling events leading to increased cell proliferation which may result into early stages of PCa. Further studies are warranted to test this hypothesis and determine the relationship between AR and ERβ2 in the presence of 27-OHC in the context of wildtype AR+ prostate cancers.

Although there are variations between the both the PCa cells due to their difference in phenotypes, they have notable similarities. Both having mutated ARs, LNCaP having a mutated AR for increased androgen sensitivity and PC3 having the mutated AR for decreased androgen sensitivity, hence each cell line depicts a different stage of PCa. Also, 27-OHC increased proliferation, decreased cell invasion and increased expression of CTGF in both cell lines, CTGF is known to play anti-metastatic roles(Chang et al. 2004; Lin et al. 2005). These results establish a rationale and prelude to the potential role of 27-OHC in promoting tumor growth in localized prostate cancers.

Altogether, our results demonstrate that 27-OHC induces an increase in cell proliferation in PCa cells. We also show for the first time that 27-OHC-induced cell proliferation is dependent on ER activation, specifically ERβ, in non-tumorigenic and PCa cells. Our study brings new insights into the potential role of 27-OHC-evoked effects on ERs in PCa development and progression. Further studies delineating underlying mechanisms involved in 27-OHC induced ER-AR crosstalk in the context of PCa are warranted and may reveal novel therapeutic avenues to prevent, delay and/or attenuate PCa progression.

CHAPTER V

CONCLUDING REMARKS AND FUTURE DIRECTIONS

27-OHC and Breast Cancer

We have demonstrated a novel cellular mechanism of action of 27-OHC which is downstream of ER and contributes to proliferation of ER+ breast cancer cells. We have shown for the first time that 27-OHC induces an increase in cell proliferation in ER+ MCF7 cells via the p53-MDM2 axis (Raza et al. 2015). Further in vivo studies are required to corroborate and apply these findings in a more clinical model such as breast cancer mouse models. Moreover, while this study establishes the importance of 27-OHC mediated ER activation in ER+ breast cancer and identifies the p53-MDM2 axis as a potential culprit, many pieces of this puzzle are missing. For instance, what are the covalent modifications of p53 and MDM2 that contribute to 27-OHC induced p53 inactivity or degradation leading to ER+ breast cancer progression? Given that 27-OHC is an abundant oxysterol in the circulation, do the oxysterol transport proteins play a role in stimulating ER downstream events leading to ER+ breast cancer progression? These questions only address part of the missing pieces that are essential to this study and its potential clinical implication towards ER+ breast cancers.

Another confounding factor to consider and further investigate is that 27-OHC is also a ligand for LXR. However, the role of LXR in the etio-pathogenesis of breast cancer is poorly understood. Nelson et.al showed that in mice with elevated plasma levels of 27-OHC, there is an increase in metastatic lesions and epithelial to mesenchymal (EMT) markers (Nelson et al. 2013). Additionally, LXR activation via LXR agonist GW3965 also increased metastasis and EMT markers (Nelson et al. 2013). However, the question whether 27-OHC induced breast cancer metastasis was LXR dependent remains unanswered, since the effect of 27-OHC on breast cancer metastasis in LXR depleted conditions was not tested. It is important to note that while 27-OHC is a ligand of both ERs and LXRs, 27-OHC is a much more efficacious ligand of ERs when compared to LXRs. 27-OHC at 1μM binds to ERβ with 90% efficacy and ERα with 50% efficacy (DuSell et al. 2008; Umetani and Shaul 2011) while 27-OHC gives a maximum of ~25-30% efficacy when bound to LXRs at 10μM (Fu et al. 2001; Spencer et al. 2001). Notably, intracellular levels of 27-OHC in macrophages can be as high as ~40 μM (Fu et al. 2001). Thus, due to 27-OHC induced concentration dependent activation of ER and LXR, it is important to quantify 27-OHC levels and delineate downstream ER-specific and LXR-specific signaling mechanisms in the presence of 27-OHC in ER+ breast cancers.

Moreover, our experiments used exogenous 27-OHC to induce ER activation leading to cell proliferation, however the endogenous availability of 27-OHC may be sufficient to stimulate cell proliferation and ER+ breast cancer growth. Interestingly, Wu et al. (2013) showed that among ER+ breast cancer

tissues analyzed, the 27-OHC metabolizing enzyme, CYP7B1 is downregulated ~50% when compared to normal breast tissue, indicating that the loss of localized 27-OHC metabolism may play a significant role in ER+ breast cancer tumor growth. Fascinatingly, upon knocking down CYP7B1 in the ER+ breast cancer cell line MCF7, without the addition of exogenous 27-OHC, cell proliferation increased by a staggering 78% (Wu et al. 2013). These results reiterate the deleterious role of tumor localized 27-OHC and perhaps the potential therapeutic significance of upregulating CYP7B1 in ER+ breast cancer tumors.

It is important to note that the widely expressed steroid hydroxylase, CYP7B1 not only metabolizes 27-OHC, but also catabolizes a number of other steroids such as dehydroepiandrosterone (DHEA), 5-androstene-3β,17β-diol (Aene-diol), 5α-androstane-3β,17β-diol (3β-Adiol) and 5α-androstane-3α,17β-diol (Lundqvist and Norlin 2012). The implications of these CYP7B1 related steroids in the context of ER+ breast cancer are also poorly understood.

Moreover, while we now know that 27-OHC exacerbates ER+ breast cancer progression, the cellular mechanism of action of 27-OHC is yet to be determined. The downstream cascade of events following 27-OHC induced ER activation that evokes ER+ breast cancer growth is poorly comprehended. A few of the signaling mechanisms involved in 27-OHC induced ER+ breast cancer growth are GDNF-RET signaling (Wu et al. 2013) and p53-MDM2 axis (Raza et al. 2015). Additionally, estradiol treated breast cancer cells regulated ~9600 genes while 27-OHC regulated ~2300 genes. Although a significant fraction of

genes regulated by estradiol and 27-OHC were the same, almost one third of genes regulated by 27-OHC treatment were not regulated by estradiol treatment (Nelson et al. 2013). This suggests the importance of studying 27-OHC- specific regulating mechanisms which may enhance our knowledge about ER+ breast cancer progression.

27-OHC and Prostate Cancer

To the best of our knowledge, we are the first to report the deleterious role of 27-OHC in the prostate. We have shown that 27-OHC increases cell proliferation of non-tumorigenic prostate epithelial cells (RWPE-1) in an ER and AR dependent manner. Given the fact that there is no evidence to suggest that 27-OHC directly binds and activates AR (Raza et al. 2016), this suggests a potential crosstalk between ER and AR in the presence of 27-OHC in the prostate. We have also shown that 27-OHC induces resistance to docetaxel induced apoptosis (Raza et al. 2016). We have also determined, for the first time, that 27-OHC induces cell proliferation in PCa cells in an ERβ dependent manner [unpublished-under peer review]. These findings warrant further experiments involving *in vivo* models testing the role of 27-OHC in PCa. Given the observation that 27-OHC increases cell proliferation in non-tumorigenic prostate epithelial cells, pending further experiments, it spotlights the tumorigenic potential of 27-OHC in the prostate.

In addition to the aforementioned limitations, these studies address and focus on only one functional component of PCa, in this case, cell proliferation. Hyperplasia is one of the beginning stages of PCa and is sustained along with the progression of the disease until metastasis occurs. Other functional metrics of prostate cancer growth such as metastasis and tumor growth have not been fully investigated due to the lack of *in vivo* resources and experimental models. Given the fact that our experiments connecting 27-OHC and PCa were the first to be reported, there is a lack of clinically relevant information that would aid our research. For instance, currently there is no information available on how much 27-OHC is found in normal versus PCa tissues. While we do know that 27-OHC levels increase with age, whether this increase also occurs in the prostate is yet to be determined.

In addition to knowing the availability of 27-OHC in the prostate, understanding the role of 27-OHC, ER and AR collectively in the context of PCa may delineate novel mechanisms involved in PCa. This knowledge may provide insights into the design of innovative and more effective therapeutic targets compared to the currently prevailing therapeutic regimens.

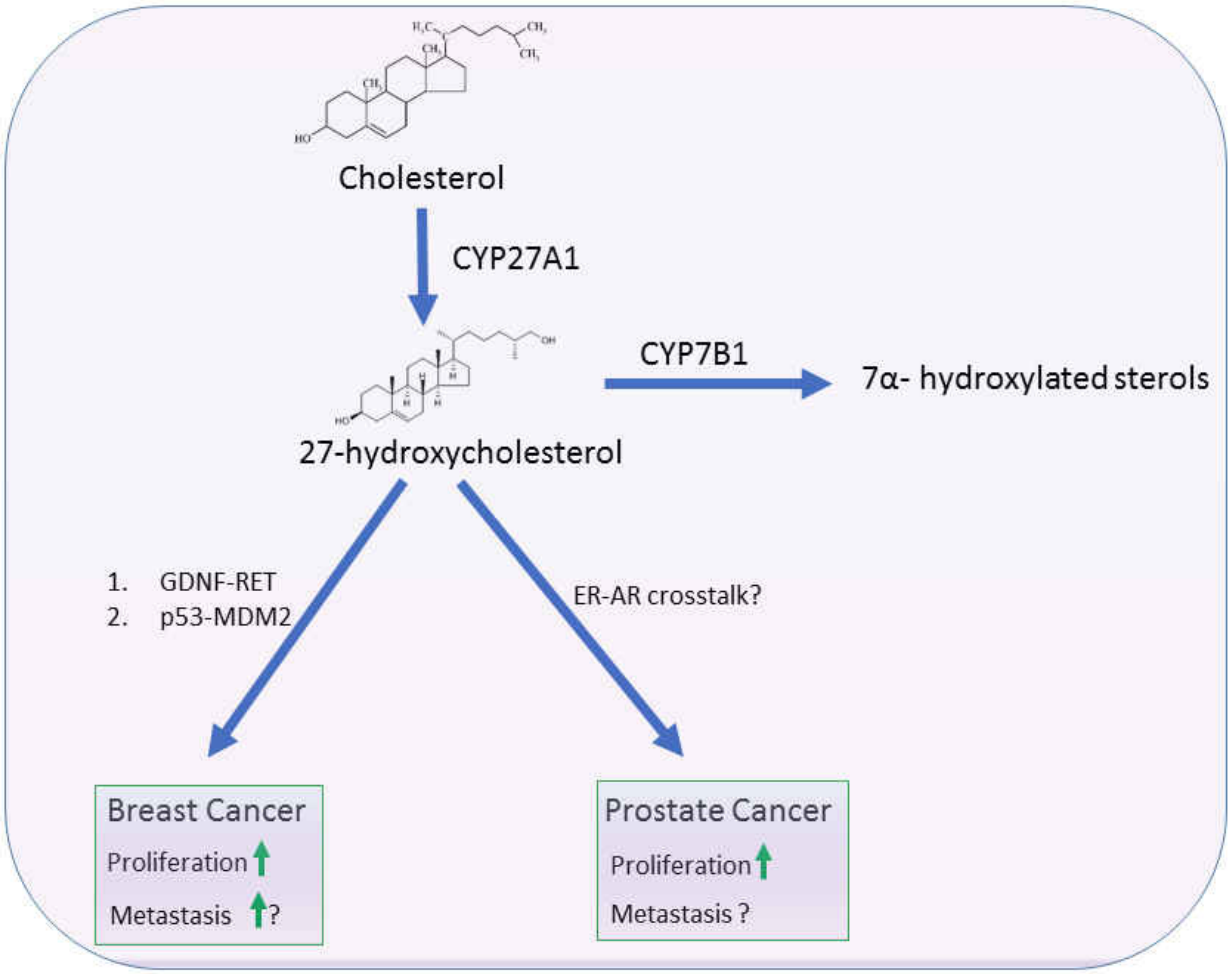


Figure 27: Schematic diagram of the role of 27-OHC in hormonal cancers. Cholesterol is converted to 27-hydroxycholesterol by CYP27A1 which exacerbates breast cancer proliferation and metastasis by either GDNF-RET signaling and/or p53-MDM2 axis. 27-hydroxycholesterol also augments prostate cancer proliferation and potentially metastasis through ER-AR crosstalk signaling. 27 hydroxycholesterol is metabolized by CYP7B1 to 7α-hydroxylated sterols.

Future Directions

The discovery that 27-OHC is an endogenous SERM has generated multiple avenues of medical research especially in the realm of endocrine related cancers. However, currently we do not fully comprehend the downstream signaling cascades involved in 27-OHC induced ER activation in endocrine related cancers. Furthermore, there is a dearth of information on genome-wide binding sites of ERs upon 27-OHC induced activation compared to estradiol mediated ER activation. Given the complexity of nuclear receptor biology and the tissue dependent promiscuous ligand binding activity of 27-OHC to ER and LXR, a clearer understanding on how 27-OHC transports and activates receptors in different tissues at various concentrations is warranted. Also, the crosstalk signaling, if any, between ER and other nuclear receptors, including AR and LXR, is yet to be elucidated in the context of hormonal cancers.

Additionally, further studies are warranted to investigate whether 27-OHC binds to the membrane bound ER, G-protein coupled estrogen receptor, GPER (GPR30). GPER plays a significant role in propagating the non-genomic pathway

of ERs and contributes to cancer progression and metastasis (Arias-Pulido et al. 2010; Scaling et al. 2014).

Understanding the mechanisms involved in 27-OHC induced ER activation endocrine related cancers can aide us in identifying novel therapeutic targets that can effectively challenge endocrine therapy resistant and recurrent hormonal cancers.

REFERENCES

Ahern TP, Pedersen L, Tarp M, Cronin-Fenton DP, Garne JP, Silliman RA, Sørensen HT, Lash TL. 2011. Statin prescriptions and breast cancer recurrence risk: a Danish nationwide prospective cohort study. J. Natl. Cancer Inst. 103:1461–8.

Ahn SH, Kim HJ, Han W, Cho J, Gong G, Jung KH, Kim S-B, Son BH, Lee JW. 2013. Effect Modification of Hormonal Therapy by p53 Status in Invasive Breast Cancer. J. Breast Cancer 16:386–94.

Alarcon-Vargas D. 2002. p53-Mdm2--the affair that never ends. Carcinogenesis 23:541–547.

Algotar AM, Behnejad R, Stratton MS, Stratton SP. 2014. Chronic use of NSAIDs and/or statins does not affect PSA or PSA velocity in men at high risk for prostate cancer. Cancer Epidemiol. Biomarkers Prev. 23:2196–8.

Ali S, Coombes RC. 2000. Estrogen receptor alpha in human breast cancer: occurrence and significance. J. Mammary Gland Biol. Neoplasia 5:271–81.

Arias-Pulido H, Royce M, Gong Y, Joste N, Lomo L, Lee S-J, Chaher N, Verschraegen C, Lara J, Prossnitz ER, et al. 2010. GPR30 and estrogen receptor expression: new insights into hormone dependence of inflammatory breast cancer. Breast Cancer Res. Treat. 123:51–8.

Arpino G, Weiss H, Lee A V, Schiff R, De Placido S, Osborne CK, Elledge RM. 2005. Estrogen receptor-positive, progesterone receptor-negative breast cancer: association with growth factor receptor expression and tamoxifen resistance. J. Natl. Cancer Inst. 97:1254–61.

Bailey ST, Shin H, Westerling T, Liu XS, Brown M. 2012. Estrogen receptor prevents p53-dependent apoptosis in breast cancer. Proc. Natl. Acad. Sci. U. S. A. 109:18060–5.

Bandaru VVR, Haughey NJ. 2014. Quantitative detection of free 24S-hydroxycholesterol, and 27-hydroxycholesterol from human serum. BMC Neurosci. 15:137.

Baum M, Budzar AU, Cuzick J, Forbes J, Houghton JH, Klijn JGM, Sahmoud T. 2002a. Anastrozole alone or in combination with tamoxifen versus tamoxifen alone for adjuvant treatment of postmenopausal women with early breast cancer: first results of the ATAC randomised trial. Lancet 359:2131–9.

Baum M, Budzar AU, Cuzick J, Forbes J, Houghton JH, Klijn JGM, Sahmoud T. 2002b. Anastrozole alone or in combination with tamoxifen versus tamoxifen alone for adjuvant treatment of postmenopausal women with early breast cancer: first results of the ATAC randomised trial. Lancet 359:2131–9.

Bello D, Webber MM, Kleinman HK, Wartinger DD, Rhim JS. 1997. Androgen responsive adult human prostatic epithelial cell lines immortalized by human papillomavirus 18. Carcinogenesis 18:1215–23.

Bereczki O, Ujfaludi Z, Pardi N, Nagy Z, Tora L, Boros IM, Balint E. 2008. TATA binding protein associated factor 3 (TAF3) interacts with p53 and inhibits its function. BMC Mol. Biol. 9:57.

Berg JM, Tymoczko JL, Stryer L. 2002. Important Derivatives of Cholesterol Include Bile Salts and Steroid Hormones.

Björkhem I. 2002. Do oxysterols control cholesterol homeostasis? J. Clin. Invest. 110:725–30.

Björkhem I. 2013. Five decades with oxysterols. Biochimie 95:448–454.

Björkhem I, Diczfalusy U. 2002. Oxysterols: friends, foes, or just fellow passengers? Arterioscler. Thromb. Vasc. Biol. 22:734–42.

Björkhem I, Lövgren-Sandblom A, Leoni V, Meaney S, Brodin L, Salveson L, Winge K, Pålhagen S, Svenningsson P. 2013. Oxysterols and Parkinson's disease: evidence that levels of 24S-hydroxycholesterol in cerebrospinal fluid correlates with the duration of the disease. Neurosci. Lett. 555:102–5.

Blows FM, Driver KE, Schmidt MK, Broeks A, van Leeuwen FE, Wesseling J, Cheang MC, Gelmon K, Nielsen TO, Blomqvist C, et al. 2010. Subtyping of breast cancer by immunohistochemistry to investigate a relationship between subtype and short and long term survival: a collaborative analysis of data for 10,159 cases from 12 studies. PLoS Med. 7:e1000279.

Bodicoat DH, Schoemaker MJ, Jones ME, McFadden E, Griffin J, Ashworth A, Swerdlow AJ. 2014. Timing of pubertal stages and breast cancer risk: the Breakthrough Generations Study. Breast Cancer Res. 16:R18.

Bonkhoff H, Fixemer T, Hunsicker I, Remberger K. 1999. Estrogen receptor expression in prostate cancer and premalignant prostatic lesions. Am. J. Pathol. 155:641–7.

Bosland MC. 2005. The role of estrogens in prostate carcinogenesis: a rationale for chemoprevention. Rev. Urol. 7 Suppl 3:S4–S10.

Bosland MC, Ford H, Horton L. 1995. Induction at high incidence of ductal prostate adenocarcinomas in NBL/Cr and Sprague-Dawley Hsd:SD rats treated with a combination of testosterone and estradiol-17 beta or diethylstilbestrol. Carcinogenesis 16:1311–7.

Bostwick DG, Burke HB, Djakiew D, Euling S, Ho S, Landolph J, Morrison H, Sonawane B, Shifflett T, Waters DJ, et al. 2004. Human prostate cancer risk factors. Cancer 101:2371–490.

Breau RH, Karnes RJ, Jacobson DJ, McGree ME, Jacobsen SJ, Nehra A, Lieber MM, St Sauver JL. 2010. The association between statin use and the diagnosis of prostate cancer in a population based cohort. J. Urol. 184:494–9.

Broustas CG, Lieberman HB. 2014. DNA damage response genes and the development of cancer metastasis. Radiat. Res. 181:111–30.

Brown AJ, Jessup W. 1999. Oxysterols and atherosclerosis. Atherosclerosis 142:1–28.

van der Burg SH, de Cock K, Menon AG, Franken KL, Palmen M, Redeker A, Drijfhout J, Kuppen PJ, van de Velde C, Erdile L, et al. 2001. Long lasting p53-specific T cell memory responses in the absence of anti-p53 antibodies in patients with resected primary colorectal cancer. Eur. J. Immunol. 31:146–55.

Burkard I, von Eckardstein A, Waeber G, Vollenweider P, Rentsch KM. 2007a. Lipoprotein distribution and biological variation of 24S- and 27-hydroxycholesterol in healthy volunteers. Atherosclerosis 194:71–8.

Burkard I, von Eckardstein A, Waeber G, Vollenweider P, Rentsch KM. 2007b. Lipoprotein distribution and biological variation of 24S- and 27-hydroxycholesterol in healthy volunteers. Atherosclerosis 194:71–8.

Burton AJ, Tilling KM, Holly JM, Hamdy FC, Rowlands M-AE, Donovan JL, Martin RM. 2010. Metabolic imbalance and prostate cancer progression. Int. J. Mol. Epidemiol. Genet. 1:248–71.

Buzdar AU. 2004. Phase III study of letrozole versus tamoxifen as first-line therapy of advanced breast cancer in postmenopausal women: analysis of survival and update of efficacy from the international letrozole breast cancer group. J. Clin. Oncol. 22:3199–200; author reply 3200–1.

Cancer Genome Atlas Network. 2012. Comprehensive molecular portraits of human breast tumours. Nature 490:61–70.

Chakravarty D, Sboner A, Nair SS, Giannopoulou E, Li R, Hennig S, Mosquera JM, Pauwels J, Park K, Kossai M, et al. 2014. The oestrogen receptor alpha-regulated lncRNA NEAT1 is a critical modulator of prostate cancer. Nat. Commun. 5:5383.

Chang C-C, Shih J-Y, Jeng Y-M, Su J-L, Lin B-Z, Chen S-T, Chau Y-P, Yang P-C, Kuo M-L. 2004. Connective Tissue Growth Factor and Its Role in Lung Adenocarcinoma Invasion and Metastasis. JNCI J. Natl. Cancer Inst. 96:364–375.

Chen M, Ni J, Chang H-C, Lin C-Y, Muyan M, Yeh S. 2009. CCDC62/ERAP75 functions as a coactivator to enhance estrogen receptor beta-mediated transactivation and target gene expression in prostate cancer cells. Carcinogenesis 30:841–50.

Chen Y, Clegg NJ, Scher HI. 2009. Anti-androgens and androgen-depleting therapies in prostate cancer: new agents for an established target. Lancet. Oncol. 10:981–91.

Chodak G. 2006. Prostate cancer: epidemiology, screening, and biomarkers. Rev. Urol. 8 Suppl 2:S3–8.

Chokkalingam AP, Nyrén O, Johansson J-E, Gridley G, McLaughlin JK, Adami H-O, Hsing AW. 2003. Prostate carcinoma risk subsequent to diagnosis of benign prostatic hyperplasia: a population-based cohort study in Sweden. Cancer 98:1727–34.

Christoforou P, Christopoulos PF, Koutsilieris M. 2014. The role of estrogen receptor β in prostate cancer. Mol. Med. 20:427–34.

Di Ciaula A, Wang DQ-H, Garruti G, Wang HH, Grattagliano I, de Bari O, Portincasa P. 2014. Therapeutic reflections in cholesterol homeostasis and gallstone disease: a review. Curr. Med. Chem. 21:1435–47.

Coates AS, Millar EK, O'Toole SA, Molloy TJ, Viale G, Goldhirsch A, Regan MM, Gelber RD, Sun Z, Castiglione-Gertsch M, et al. 2012. Prognostic interaction between expression of p53 and estrogen receptor in patients with node-negative breast cancer: results from IBCSG Trials VIII and IX. Breast Cancer Res. 14:R143.

Compton DR, Sheng S, Carlson KE, Rebacz NA, Lee IY, Katzenellenbogen BS, Katzenellenbogen JA. 2004. Pyrazolo[1,5-a]pyrimidines: estrogen receptor ligands possessing estrogen receptor beta antagonist activity. J. Med. Chem. 47:5872–93.

Comşa Ş, Cîmpean AM, Raica M. 2015. The Story of MCF-7 Breast Cancer Cell Line: 40 years of Experience in Research. Anticancer Res. 35:3147–54.

Crujeiras AB, Díaz-Lagares A, Carreira MC, Amil M, Casanueva FF. 2013. Oxidative stress associated to dysfunctional adipose tissue: a potential link between obesity, type 2 diabetes mellitus and breast cancer. Free Radic. Res. 47:243–56.

Cruz P, Epuñán MJ, Ramírez ME, Torres CG, Valladares LE, Sierralta WD. 2012. 27-hydroxycholesterol and the expression of three estrogen-sensitive proteins in MCF7 cells. Oncol. Rep. 28:992–998.

Cruz P, Torres C, Ramírez ME, Epuñán MJ, Valladares LE, Sierralta WD. 2010. Proliferation of human mammary cancer cells exposed to 27-hydroxycholesterol. Exp. Ther. Med. 1:531–536.

Cruz P, Torres C, Ramírez ME, Epuñán MJ, Valladares LE, Sierralta WD. 2010. Proliferation of human mammary cancer cells exposed to 27-hydroxycholesterol. Exp. Ther. Med. 1:531–536.

Dai X, Fang X, Ma Y, Xianyu J. 2016. Benign Prostatic Hyperplasia and the Risk of Prostate Cancer and Bladder Cancer: A Meta-Analysis of Observational Studies. Medicine (Baltimore). 95:e3493.

Dasari B, Prasanthi JRP, Marwarha G, Singh BB, Ghribi O. 2010. The oxysterol 27-hydroxycholesterol increases β-amyloid and oxidative stress in retinal pigment epithelial cells. BMC Ophthalmol. 10:22.

Delongchamps NB, Singh A, Haas GP. 2006. The role of prevalence in the diagnosis of prostate cancer. Cancer Control 13:158–68.

Dhingra N, Bhagwat D. 2011. Benign prostatic hyperplasia: An overview of existing treatment. Indian J. Pharmacol. 43:6–12.

Dietschy JM. 1984. Regulation of cholesterol metabolism in man and in other species. Klin. Wochenschr. 62:338–345.

Doisneau-Sixou SF, Sergio CM, Carroll JS, Hui R, Musgrove EA, Sutherland RL. 2003. Estrogen and antiestrogen regulation of cell cycle progression in breast cancer cells. Endocr. Relat. Cancer 10:179–86.

Dolfi SC, Jäger A V, Medina DJ, Haffty BG, Yang J-M, Hirshfield KM. 2014. Fulvestrant treatment alters MDM2 protein turnover and sensitivity of human breast carcinoma cells to chemotherapeutic drugs. Cancer Lett. 350:52–60.

Duane WC, Javitt NB. 1999. 27-Hydroxycholesterol: production rates in normal human subjects. J. Lipid Res. 40:1194–1199.

DuSell CD, Nelson ER, Wang X, Abdo J, Mödder UI, Umetani M, Gesty-Palmer D, Javitt NB, Khosla S, McDonnell DP. 2010. The endogenous selective estrogen receptor modulator 27-hydroxycholesterol is a negative regulator of bone homeostasis. Endocrinology 151:3675–85.

DuSell CD, Umetani M, Shaul PW, Mangelsdorf DJ, McDonnell DP. 2008. 27-hydroxycholesterol is an endogenous selective estrogen receptor modulator. Mol. Endocrinol. 22:65–77.

Dzeletovic S, Breuer O, Lund E, Diczfalusy U. 1995. Determination of cholesterol oxidation products in human plasma by isotope dilution-mass spectrometry. Anal. Biochem. 225:73–80.

Ferlay J, Shin H-R, Bray F, Forman D, Mathers C, Parkin DM. 2010. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int. J. Cancer 127:2893–917.

Franco S, Perez A, Tan-Chiu E, Frankel C, Vogel CL. 2004. Response to fulvestrant in heavily pretreated postmenopausal women: a single-center experience. Breast Cancer Res. Treat. 88:103–8.

Fu X, Menke JG, Chen Y, Zhou G, MacNaul KL, Wright SD, Sparrow CP, Lund EG. 2001. 27-Hydroxycholesterol Is an Endogenous Ligand for Liver X Receptor in Cholesterol-loaded Cells. J. Biol. Chem. 276:38378–38387.

Gabitova L, Gorin A, Astsaturov I. 2013. Molecular Pathways: Sterols and Receptor Signaling in Cancer. Clin. Cancer Res. 20:28–34.

Gann PH. 2002. Risk factors for prostate cancer. Rev. Urol. 4 Suppl 5:S3–S10.

Gao C-F, Xie Q, Su Y-L, Koeman J, Khoo SK, Gustafson M, Knudsen BS, Hay R, Shinomiya N, Vande Woude GF. 2005. Proliferation and invasion: plasticity in tumor cells. Proc. Natl. Acad. Sci. U. S. A. 102:10528–33.

Garnick MB, Fair WR. 1998. Combating prostate cancer. Sci. Am. 279:74–83.

Geybels MS, Wright JL, Holt SK, Kolb S, Feng Z, Stanford JL. 2013. Statin use in relation to prostate cancer outcomes in a population-based patient cohort study. Prostate 73:1214–22.

Giaccia AJ, Kastan MB. 1998. The complexity of p53 modulation: emerging patterns from divergent signals. Genes Dev. 12:2973–2983.

Goedeke L, Fernández-Hernando C. 2012. Regulation of cholesterol homeostasis. Cell. Mol. Life Sci. 69:915–30.

Gómez-Suaga P, Fdez E, Fernández B, Martínez-Salvador M, Blanca Ramírez M, Madero-Pérez J, Rivero-Ríos P, Fuentes JM, Hilfiker S. 2014. Novel insights into the neurobiology underlying LRRK2-linked Parkinson's disease. Neuropharmacology 85C:45–56.

Gonzalez-Angulo AM, Morales-Vasquez F, Hortobagyi GN. 2007. Overview of resistance to systemic therapy in patients with breast cancer. Adv. Exp. Med. Biol. 608:1–22.

Gorlov IP, Sircar K, Zhao H, Maity SN, Navone NM, Gorlova OY, Troncoso P, Pettaway CA, Byun JY, Logothetis CJ. 2010. Prioritizing genes associated with prostate cancer development. BMC Cancer 10:599.

Grundy SM, Bilheimer DW. 1989. Cholesterol education implications for clinical practice. Tex. Med. 85:23–7.

Guess HA, Arrighi HM, Metter EJ, Fozard JL. 1990. Cumulative prevalence of prostatism matches the autopsy prevalence of benign prostatic hyperplasia. Prostate 17:241–6.

Guven GS, Atalar E, Yavuz B, Beyazit Y, Kekilli M, Kilicarslan A, Sahiner L, Oz G, Ozer N, Aksoyek S, et al. 2006. Simvastatin treatment improves endothelial function and increases fibrinolysis in patients with hypercholestrolemia. J. Natl. Med. Assoc. 98:627–30.

Guyatt GH, Oxman AD, Kunz R, Vist GE, Falck-Ytter Y, Schünemann HJ. 2008. What is "quality of evidence" and why is it important to clinicians? BMJ 336:995–8.

Hall JM, Couse JF, Korach KS. 2001. The Multifaceted Mechanisms of Estradiol and Estrogen Receptor Signaling. J. Biol. Chem. 276:36869–36872.

Hanahan D, Weinberg RA. 2000. The Hallmarks of Cancer. Cell 100:57–70.

Harrell JC, Dye WW, Allred DC, Jedlicka P, Spoelstra NS, Sartorius CA, Horwitz KB. 2006. Estrogen receptor positive breast cancer metastasis: altered hormonal sensitivity and tumor aggressiveness in lymphatic vessels and lymph nodes. Cancer Res. 66:9308–15.

Hart CD, Migliaccio I, Malorni L, Guarducci C, Biganzoli L, Di Leo A. 2015. Challenges in the management of advanced, ER-positive, HER2-negative breast cancer. Nat. Rev. Clin. Oncol. 12:541–52.

Hartman J, Ström A, Gustafsson J-Å. 2012. Current concepts and significance of estrogen receptor β in prostate cancer. Steroids 77:1262–6.

Hasson SP, Rubinek T, Ryvo L, Wolf I. 2013. Endocrine Resistance in Breast Cancer: Focus on the Phosphatidylinositol 3-Kinase/Akt/Mammalian Target of Rapamycin Signaling Pathway. Breast Care (Basel). 8:248–255.

Heinlein CA, Chang C. 2004. Androgen receptor in prostate cancer. Endocr. Rev. 25:276–308.

Higgins JPT, Thompson SG. 2002. Quantifying heterogeneity in a meta-analysis. Stat. Med. 21:1539–58.

Hipfner DR, Cohen SM. 2004. Connecting proliferation and apoptosis in development and disease. Nat. Rev. Mol. Cell Biol. 5:805–15.

Hirayama T, Mizokami Y, Honda A, Homma Y, Ikegami T, Saito Y, Miyazaki T, Matsuzaki Y. 2009. Serum concentration of 27-hydroxycholesterol predicts the effects of high-cholesterol diet on plasma LDL cholesterol level. Hepatol. Res. 39:149–56.

Horie-Inoue K, Bono H, Okazaki Y, Inoue S. 2004. Identification and functional analysis of consensus androgen response elements in human prostate cancer cells. Biochem. Biophys. Res. Commun. 325:1312–7.

Hwang C. 2012. Overcoming docetaxel resistance in prostate cancer: a perspective review. Ther. Adv. Med. Oncol. 4:329–40.

Imamov O, Morani A, Shim G-J, Omoto Y, Thulin-Andersson C, Warner M, Gustafsson J-A. 2004. Estrogen receptor beta regulates epithelial cellular differentiation in the mouse ventral prostate. Proc. Natl. Acad. Sci. U. S. A. 101:9375–80.

Izumi K, Mizokami A, Lin W-J, Lai K-P, Chang C. 2013. Androgen receptor roles in the development of benign prostate hyperplasia. Am. J. Pathol. 182:1942–9.

Jacobson A, Cunningham JL. 2012. Connective tissue growth factor in tumor pathogenesis. Fibrogenesis Tissue Repair 5:S8.

James FR, Wootton S, Jackson A, Wiseman M, Copson ER, Cutress RI. 2015. Obesity in breast cancer--what is the risk factor? Eur. J. Cancer 51:705–20.

Jankowitz RC, Davidson NE. 2013. Adjuvant endocrine therapy for breast cancer: how long is long enough? Oncology (Williston Park). 27:1210–6, 1224.

Javitt NB. 2008. Oxysterols: novel biologic roles for the 21st century. Steroids 73:149–57.

Jedinak A, Curatolo A, Zurakowski D, Dillon S, Bhasin MK, Libermann TA, Roy R, Sachdev M, Loughlin KR, Moses MA. 2015. Novel non-invasive biomarkers that distinguish between benign prostate hyperplasia and prostate cancer. BMC Cancer 15:259.

Jones SN, Hancock AR, Vogel H, Donehower LA, Bradley A. 1998. Overexpression of Mdm2 in mice reveals a p53-independent role for Mdm2 in tumorigenesis. Proc. Natl. Acad. Sci. 95:15608–15612.

Kaarbø M, Klokk TI, Saatcioglu F. 2007. Androgen signaling and its interactions with other signaling pathways in prostate cancer. Bioessays 29:1227–38.

Kaighn ME, Narayan KS, Ohnuki Y, Lechner JF, Jones LW. 1979. Establishment and characterization of a human prostatic carcinoma cell line (PC-3). Invest. Urol. 17:16–23.

Kantor ED, Lipworth L, Fowke JH, Giovannucci EL, Mucci LA, Signorello LB. 2015. Statin use and risk of prostate cancer: Results from the Southern Community Cohort Study. Prostate 75:1384–93.

Karanika S, Karantanos T, Kurosaka S, Wang J, Hirayama T, Yang G, Park S, Golstov AA, Tanimoto R, Li L, et al. 2015. GLIPR1-ΔTM synergizes with docetaxel in cell death and suppresses resistance to docetaxel in prostate cancer cells. Mol. Cancer 14:122.

Karuna R, Holleboom AG, Motazacker MM, Kuivenhoven JA, Frikke-Schmidt R, Tybjaerg-Hansen A, Georgopoulos S, van Eck M, van Berkel TJC, von Eckardstein A, et al. 2011. Plasma levels of 27-hydroxycholesterol in humans and mice with monogenic disturbances of high density lipoprotein metabolism. Atherosclerosis 214:448–55.

Kellokumpu-Lehtinen P-L, Harmenberg U, Joensuu T, McDermott R, Hervonen P, Ginman C, Luukkaa M, Nyandoto P, Hemminki A, Nilsson S, et al. 2013. 2-Weekly versus 3-weekly docetaxel to treat castration-resistant advanced prostate cancer: a randomised, phase 3 trial. Lancet. Oncol. 14:117–24.

Khandrika L, Kumar B, Koul S, Maroni P, Koul HK. 2009. Oxidative stress in prostate cancer. Cancer Lett. 282:125–36.

Khoo KH, Hoe KK, Verma CS, Lane DP. 2014. Drugging the p53 pathway: understanding the route to clinical efficacy. Nat. Rev. Drug Discov. 13:217–36.

Kim J, Coetzee GA. 2004. Prostate specific antigen gene regulation by androgen receptor. J. Cell. Biochem. 93:233–41.

King M. 2016. Cholesterol: Synthesis, Metabolism, Regulation. [accessed 2013 Apr 23]. http://themedicalbiochemistrypage.org/cholesterol.php

Krakhmal N V, Zavyalova M V, Denisov E V, Vtorushin S V, Perelmuter VM. 2015. Cancer Invasion: Patterns and Mechanisms. Acta Naturae 7:17–28.

Kruse J-P, Gu W. 2009. Modes of p53 regulation. Cell 137:609–22.

Kun Y, How LC, Hoon TP, Bajic VB, Lam TS, Aggarwal A, Sze HG, Bok WS, Yin WC, Tan P. 2003. Classifying the estrogen receptor status of breast cancers by expression profiles reveals a poor prognosis subpopulation exhibiting high expression of the ERBB2 receptor. Hum. Mol. Genet. 12:3245–58.

Lain S, Hollick JJ, Campbell J, Staples OD, Higgins M, Aoubala M, McCarthy A, Appleyard V, Murray KE, Baker L, et al. 2008. Discovery, in vivo activity, and mechanism of action of a small-molecule p53 activator. Cancer Cell 13:454–63.

Lee A V, Oesterreich S, Davidson NE. 2015. MCF-7 cells--changing the course of breast cancer research and care for 45 years. J. Natl. Cancer Inst. 107:djv073–.

Lee SH, Park TJ, Bae MH, Choi SH, Cho YS, Joo KJ, Kwon CH, Park HJ. 2013. Impact of treatment with statins on prostate-specific antigen and prostate volume in patients with benign prostatic hyperplasia. Korean J. Urol. 54:750–5.

Lehmann JM, Kliewer SA, Moore LB, Smith-Oliver TA, Oliver BB, Su J-L, Sundseth SS, Winegar DA, Blanchard DE, Spencer TA, et al. 1997. Activation of the Nuclear Receptor LXR by Oxysterols Defines a New Hormone Response Pathway. J. Biol. Chem. 272:3137–3140.

Leung Y-K, Lam H-M, Wu S, Song D, Levin L, Cheng L, Wu C-L, Ho S-M. 2010. Estrogen receptor beta2 and beta5 are associated with poor prognosis in prostate cancer, and promote cancer cell migration and invasion. Endocr. Relat. Cancer 17:675–89.

Lim LY, Vidnovic N, Ellisen LW, Leong C-O. 2009. Mutant p53 mediates survival of breast cancer cells. Br. J. Cancer 101:1606–12.

Lin B-R, Chang C-C, Che T-F, Chen S-T, Chen RJ-C, Yang C-Y, Jeng Y-M, Liang J-T, Lee P-H, Chang K-J, et al. 2005. Connective tissue growth factor inhibits metastasis and acts as an independent prognostic marker in colorectal cancer. Gastroenterology 128:9–23.

Lonergan PE, Tindall DJ. 2011. Androgen receptor signaling in prostate cancer development and progression. J. Carcinog. 10:20.

Lu T, Lin W-J, Izumi K, Wang X, Xu D, Fang L-Y, Li L, Jiang Q, Jin J, Chang C. 2012. Targeting androgen receptor to suppress macrophage-induced EMT and benign prostatic hyperplasia (BPH) development. Mol. Endocrinol. 26:1707–15.

Lucas JM, True L, Hawley S, Matsumura M, Morrissey C, Vessella R, Nelson PS. 2008. The androgen-regulated type II serine protease TMPRSS2 is differentially expressed and mislocalized in prostate adenocarcinoma. J. Pathol. 215:118–25.

Lundqvist J, Norlin M. 2012. Effects of CYP7B1-related steroids on androgen receptor activation in different cell lines. Biochim. Biophys. Acta 1821:973–9.

Ma D, Liu W, Wang Y. 2014 May 1. ApoA-I or ABCA1 expression suppresses fatty acid synthesis by reducing 27-hydroxycholesterol levels. Biochimie.

Magura L, Blanchard R, Hope B, Beal JR, Schwartz GG, Sahmoun AE. 2008. Hypercholesterolemia and prostate cancer: a hospital-based case-control study. Cancer Causes Control 19:1259–66.

Manfredi JJ. 2010. The Mdm2-p53 relationship evolves: Mdm2 swings both ways as an oncogene and a tumor suppressor. Genes Dev. 24:1580–9.

Marwarha G, Ghribi O. 2015. Does the oxysterol 27-hydroxycholesterol underlie Alzheimer's disease-Parkinson's disease overlap? Exp. Gerontol. 68:13–8.

Marwarha G, Rhen T, Schommer T, Ghribi O. 2011. The oxysterol 27-hydroxycholesterol regulates α-synuclein and tyrosine hydroxylase expression levels in human neuroblastoma cells through modulation of liver X receptors and estrogen receptors--relevance to Parkinson's disease. J. Neurochem. 119:1119–36.

McIlwain DR, Berger T, Mak TW. 2013. Caspase functions in cell death and disease. Cold Spring Harb. Perspect. Biol. 5:a008656.

McKeage K, Curran MP, Plosker GL. 2004. Fulvestrant: a review of its use in hormone receptor-positive metastatic breast cancer in postmenopausal women with disease progression following antiestrogen therapy. Drugs 64:633–48.

McKeage K, Keam SJ. 2005. Docetaxel in hormone-refractory metastatic prostate cancer. Drugs 65:2287–94; discussion 2295–7.

McPherson SJ, Ellem SJ, Risbridger GP. 2008. Estrogen-regulated development and differentiation of the prostate. Differentiation. 76:660–70.

Meaney S, Hassan M, Sakinis A, Lütjohann D, von Bergmann K, Wennmalm A, Diczfalusy U, Björkhem I. 2001. Evidence that the major oxysterols in human circulation originate from distinct pools of cholesterol: a stable isotope study. J. Lipid Res. 42:70–8.

Meaney S, Lütjohann D, Diczfalusy U, Björkhem I. 2000. Formation of oxysterols from different pools of cholesterol as studied by stable isotope technique: cerebral origin of most circulating 24S-hydroxycholesterol in rats, but not in mice. Biochim. Biophys. Acta 1486:293–8.

Mhaidat NM, Thorne RF, Zhang XD, Hersey P. 2007. Regulation of docetaxel-induced apoptosis of human melanoma cells by different isoforms of protein kinase C. Mol. Cancer Res. 5:1073–81.

Miller LD, Smeds J, George J, Vega VB, Vergara L, Ploner A, Pawitan Y, Hall P, Klaar S, Liu ET, et al. 2005. An expression signature for p53 status in human breast cancer predicts mutation status, transcriptional effects, and patient survival. Proc. Natl. Acad. Sci. U. S. A. 102:13550–5.

Miyachi M, Kakazu N, Yagyu S, Katsumi Y, Tsubai-Shimizu S, Kikuchi K, Tsuchiya K, Iehara T, Hosoi H. 2009. Restoration of p53 pathway by nutlin-3 induces cell cycle arrest and apoptosis in human rhabdomyosarcoma cells. Clin. Cancer Res. 15:4077–84.

Miyazawa K, Tanaka T, Nakai D, Morita N, Suzuki K. 2014. Immunohistochemical expression of four different stem cell markers in prostate cancer: High expression of NANOG in conjunction with hypoxia-inducible factor-1α expression is involved in prostate epithelial malignancy. Oncol. Lett. 8:985–992.

Moll UM, Petrenko O. 2003. The MDM2-p53 Interaction. Mol. Cancer Res. 1:1001–1008.

Momand J. 1998. The MDM2 gene amplification database. Nucleic Acids Res. 26:3453–3459.

Mondul AM, Clipp SL, Helzlsouer KJ, Platz EA. 2010. Association between plasma total cholesterol concentration and incident prostate cancer in the CLUE II cohort. Cancer Causes Control 21:61–8.

Moon H, Ruelcke JE, Choi E, Sharpe LJ, Nassar ZD, Bielefeldt-Ohmann H, Parat M-O, Shah A, Francois M, Inder KL, et al. 2015. Diet-induced hypercholesterolemia promotes androgen-independent prostate cancer metastasis via IQGAP1 and caveolin-1. Oncotarget 6:7438–7453.

Nelles JL, Hu W-Y, Prins GS. 2011. Estrogen action and prostate cancer. Expert Rev. Endocrinol. Metab. 6:437–451.

Nelson AW, Tilley WD, Neal DE, Carroll JS. 2014. Estrogen receptor beta in prostate cancer: friend or foe? Endocr. Relat. Cancer 21:T219–34.

Nelson ER, DuSell CD, Wang X, Howe MK, Evans G, Michalek RD, Umetani M, Rathmell JC, Khosla S, Gesty-Palmer D, et al. 2011. The oxysterol, 27-hydroxycholesterol, links cholesterol metabolism to bone homeostasis through its actions on the estrogen and liver X receptors. Endocrinology 152:4691–705.

Nelson ER, Wardell SE, Jasper JS, Park S, Suchindran S, Howe MK, Carver NJ, Pillai R V, Sullivan PM, Sondhi V, et al. 2013. 27-Hydroxycholesterol links hypercholesterolemia and breast cancer pathophysiology. Science 342:1094–8.

Obermiller PS, Tait DL, Holt JT. 2000. Gene therapy for carcinoma of the breast: Therapeutic genetic correction strategies. Breast Cancer Res 2:28–31.

Okumura N, Saji S, Eguchi H, Hayashi S, Saji S, Nakashima S. 2002. Estradiol Stabilizes p53 Protein in Breast Cancer Cell Line, MCF-7. Japanese J. Cancer Res. 93:867–873.

Olkkonen VM, Béaslas O, Nissilä E. 2012. Oxysterols and their cellular effectors. Biomolecules 2:76–103.

Olkkonen VM, Béaslas O, Nissilä E. 2012. Oxysterols and Their Cellular Effectors. Biomolecules 2:76–103.

Ørsted DD, Bojesen SE. 2013. The link between benign prostatic hyperplasia and prostate cancer. Nat. Rev. Urol. 10:49–54.

Papadopoulos G, Delakas D, Nakopoulou L, Kassimatis T. 2011. Statins and prostate cancer: molecular and clinical aspects. Eur. J. Cancer 47:819–30.

Park EJ, Choi KS, Yoo YH, Kwon TK. 2013. Nutlin-3, a small-molecule MDM2 inhibitor, sensitizes Caki cells to TRAIL-induced apoptosis through p53-mediated PUMA upregulation and ROS-mediated DR5 upregulation. Anticancer. Drugs 24:260–9.

Petrylak DP. 2000. Docetaxel (Taxotere) in hormone-refractory prostate cancer. Semin. Oncol. 27:24–9.

Petrylak DP. 2006. The treatment of hormone-refractory prostate cancer: docetaxel and beyond. Rev. Urol. 8 Suppl 2:S48–55.

Pfaffl MW. 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29:e45.

Pharoah PD, Day NE, Caldas C. 1999. Somatic mutations in the p53 gene and prognosis in breast cancer: a meta-analysis. Br. J. Cancer 80:1968–73.

Porter AG, Jänicke RU. 1999. Emerging roles of caspase-3 in apoptosis. Cell Death Differ. 6:99–104.

Poyurovsky M V, Katz C, Laptenko O, Beckerman R, Lokshin M, Ahn J, Byeon I-JL, Gabizon R, Mattia M, Zupnick A, et al. 2010. The C terminus of p53 binds the N-terminal domain of MDM2. Nat. Struct. Mol. Biol. 17:982–9.

Prasanthi JRP, Larson T, Schommer J, Ghribi O. 2011. Silencing GADD153/CHOP gene expression protects against Alzheimer's disease-like pathology induced by 27-hydroxycholesterol in rabbit hippocampus. PLoS One 6:e26420.

Prat A, Perou CM, Agus DB, Akita RW, Fox WD, Lewis GD, Higgins B, Pisacane PI, Lofgren JA, Tindell C, et al. 2011. Deconstructing the molecular portraits of breast cancer. Mol. Oncol. 5:5–23.

Printz C. 2014. Researchers find link between high cholesterol and breast cancer. Cancer 120:3429.

Ramirez DMO, Andersson S, Russell DW. 2008. Neuronal expression and subcellular localization of cholesterol 24-hydroxylase in the mouse brain. J. Comp. Neurol. 507:1676–93.

Rantham Prabhakara JP, Feist G, Thomasson S, Thompson A, Schommer E, Ghribi O. 2008. Differential effects of 24-hydroxycholesterol and 27-hydroxycholesterol on tyrosine hydroxylase and alpha-synuclein in human neuroblastoma SH-SY5Y cells. J. Neurochem. 107:1722–9.

Raza S, Meyer M, Schommer J, Hammer KDP, Guo B, Ghribi O. 2016. 27-Hydroxycholesterol stimulates cell proliferation and resistance to docetaxel-induced apoptosis in prostate epithelial cells. Med. Oncol. 33:12.

Raza S, Ohm JE, Dhasarathy A, Schommer J, Roche C, Hammer KDP, Ghribi O. 2015. The cholesterol metabolite 27-hydroxycholesterol regulates p53 activity and increases cell proliferation via MDM2 in breast cancer cells. Mol. Cell. Biochem. 410:187–95.

Rejeeth C, Kannan S. 2014 May 4. p53 gene therapy of human breast carcinoma: using a transferrin-modified silica nanoparticles. Breast Cancer.

Renoir J-M, Marsaud V, Lazennec G. 2012. Estrogen receptor signaling as a target for novel breast cancer therapeutics. Biochem. Pharmacol.

Rivenbark AG, O'Connor SM, Coleman WB. 2013. Molecular and cellular heterogeneity in breast cancer: challenges for personalized medicine. Am. J. Pathol. 183:1113–24.

Roehrborn CG, Spann ME, Myers SL, Serviss CR, Hu L, Jin Y. 2015. Estrogen receptor beta agonist LY500307 fails to improve symptoms in men with enlarged prostate secondary to benign prostatic hypertrophy. Prostate Cancer Prostatic Dis. 18:43–8.

Róg T, Vattulainen I. 2014. Cholesterol, sphingolipids, and glycolipids: what do we know about their role in raft-like membranes? Chem. Phys. Lipids 184:82–104.

Sasaki R, Shirakawa T, Zhang ZJ, Tamekane A, Matsumoto A, Sugimura K, Matsuo M, Kamidono S, Gotoh A. 2001. Additional gene therapy with Ad5CMV-p53 enhanced the efficacy of radiotherapy in human prostate cancer cells. Int. J. Radiat. Oncol. Biol. Phys. 51:1336–45.

Sato N, Gleave ME, Bruchovsky N, Rennie PS, Beraldi E, Sullivan LD. 1997. A Metastatic and Androgen-sensitive Human Prostate Cancer Model Using Intraprostatic Inoculation of LNCaP Cells in SCID Mice. Cancer Res. 57:1584–1589.

Sato R. 2010. Sterol metabolism and SREBP activation. Arch. Biochem. Biophys. 501:177–81.

Saxena P, Trerotola M, Wang T, Li J, Sayeed A, Vanoudenhove J, Adams DS, Fitzgerald TJ, Altieri DC, Languino LR. 2012. PSA regulates androgen receptor expression in prostate cancer cells. Prostate 72:769–76.

Scaling AL, Prossnitz ER, Hathaway HJ. 2014. GPER mediates estrogen-induced signaling and proliferation in human breast epithelial cells and normal and malignant breast. Horm. Cancer 5:146–60.

Schüle R, Siddique T, Deng H-X, Yang Y, Donkervoort S, Hansson M, Madrid RE, Siddique N, Schöls L, Björkhem I. 2010. Marked accumulation of 27-hydroxycholesterol in SPG5 patients with hereditary spastic paresis. J. Lipid Res. 51:819–23.

Seligson DB, Yu H, Tze S, Said J, Pantuck AJ, Cohen P, Lee K-W. 2013. IGFBP-3 nuclear localization predicts human prostate cancer recurrence. Horm. Cancer 4:12–23.

Shao ZM, Shen ZZ, Fontana JA, Barsky SH. Genistein's "ER-dependent and independent" actions are mediated through ER pathways in ER-positive breast carcinoma cell lines. Anticancer Res. 20:2409–16.

SHERR C. 2004. Principles of Tumor Suppression. Cell 116:235–246.

Sherr CJ, McCormick F. 2002. The RB and p53 pathways in cancer. Cancer Cell 2:103–112.

Shukla GC, Plaga AR, Shankar E, Gupta S. 2016. Androgen receptor-related diseases: what do we know? Andrology 4:366–381.

Singh P, Saxena R, Srinivas G, Pande G, Chattopadhyay A. 2013. Cholesterol biosynthesis and homeostasis in regulation of the cell cycle. PLoS One 8:e58833.

Song SU, Boyce FM. 2001. Combination treatment for osteosarcoma with baculoviral vector mediated gene therapy (p53) and chemotherapy (adriamycin). Exp. Mol. Med. 33:46–53.

Sonmez A, Yilmaz MI, Saglam M, Unal HU, Gok M, Cetinkaya H, Karaman M, Haymana C, Eyileten T, Oguz Y, et al. 2015. The role of plasma triglyceride/high-density lipoprotein cholesterol ratio to predict cardiovascular outcomes in chronic kidney disease. Lipids Health Dis. 14:29.

Sørlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, Hastie T, Eisen MB, van de Rijn M, Jeffrey SS, et al. 2001. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc. Natl. Acad. Sci. U. S. A. 98:10869–74.

Speetjens FM, Kuppen PJK, Welters MJP, Essahsah F, Voet van den Brink AMEG, Lantrua MGK, Valentijn ARPM, Oostendorp J, Fathers LM, Nijman HW, et al. 2009. Induction of p53-specific immunity by a p53 synthetic long peptide vaccine in patients treated for metastatic colorectal cancer. Clin. Cancer Res. 15:1086–95.

Spencer TA, Li D, Russel JS, Collins JL, Bledsoe RK, Consler TG, Moore LB, Galardi CM, McKee DD, Moore JT, et al. 2001. Pharmacophore analysis of the nuclear oxysterol receptor LXRalpha. J. Med. Chem. 44:886–97.

Tai S, Sun Y, Squires JM, Zhang H, Oh WK, Liang C-Z, Huang J. 2011. PC3 is a cell line characteristic of prostatic small cell carcinoma. Prostate 71:1668–79.

Tsvetkov P, Reuven N, Shaul Y. 2010. Ubiquitin-independent p53 proteasomal degradation. Cell Death Differ. 17:103–8.

Umetani M, Domoto H, Gormley AK, Yuhanna IS, Cummins CL, Javitt NB, Korach KS, Shaul PW, Mangelsdorf DJ. 2007a. 27-Hydroxycholesterol is an endogenous SERM that inhibits the cardiovascular effects of estrogen. Nat. Med. 13:1185–92.

Umetani M, Domoto H, Gormley AK, Yuhanna IS, Cummins CL, Javitt NB, Korach KS, Shaul PW, Mangelsdorf DJ. 2007b. 27-Hydroxycholesterol is an endogenous SERM that inhibits the cardiovascular effects of estrogen. Nat. Med. 13:1185–92.

Umetani M, Ghosh P, Ishikawa T, Umetani J, Ahmed M, Mineo C, Shaul PW. 2014. The cholesterol metabolite 27-hydroxycholesterol promotes atherosclerosis via proinflammatory processes mediated by estrogen receptor alpha. Cell Metab. 20:172–82.

Umetani M, Shaul PW. 2011. 27-Hydroxycholesterol: the first identified endogenous SERM. Trends Endocrinol. Metab. 22:130–5.

Veldscholte J, Berrevoets CA, Ris-Stalpers C, Kuiper GG, Jenster G, Trapman J, Brinkmann AO, Mulder E. 1992. The androgen receptor in LNCaP cells contains a mutation in the ligand binding domain which affects steroid binding characteristics and response to antiandrogens. J. Steroid Biochem. Mol. Biol. 41:665–9.

Vurusaner B, Leonarduzzi G, Gamba P, Poli G, Basaga H. 2016 Mar. Oxysterols and mechanisms of survival signaling. Mol. Aspects Med.

Walerych D, Napoli M, Collavin L, Del Sal G. 2012. The rebel angel: mutant p53 as the driving oncogene in breast cancer. Carcinogenesis 33:2007–17.

Wang H, Zeng X, Oliver P, Le LP, Chen J, Chen L, Zhou W, Agrawal S, Zhang R. 1999. MDM2 oncogene as a target for cancer therapy: An antisense approach. Int. J. Oncol. 15:653–60.

Wang Y, Shi J, Chai K, Ying X, Zhou BP. 2013. The Role of Snail in EMT and Tumorigenesis. Curr. Cancer Drug Targets 13:963–72.

Wasielewski M, Elstrodt F, Klijn JGM, Berns EMJJ, Schutte M. 2006. Thirteen new p53 gene mutants identified among 41 human breast cancer cell lines. Breast Cancer Res. Treat. 99:97–101.

Weihua Z, Lathe R, Warner M, Gustafsson J-A. 2002. An endocrine pathway in the prostate, ERbeta, AR, 5alpha-androstane-3beta,17beta-diol, and CYP7B1, regulates prostate growth. Proc. Natl. Acad. Sci. U. S. A. 99:13589–94.

White CP. 1909. On the occurrence of crystals in tumours. J. Pathol. Bacteriol. 13:3–10.

William G. Nelson, M.D., Ph.D., Angelo M. De Marzo, M.D., Ph.D., and William B. Isaacs PD. 2003. Prostate Cancer. N Engl J Med:366–381. [accessed 2014 Oct 6]. http://www.nejm.org/doi/full/10.1056/NEJMra021562

Wu Q, Ishikawa T, Sirianni R, Tang H, McDonald JG, Yuhanna IS, Thompson B, Girard L, Mineo C, Brekken RA, et al. 2013. 27-Hydroxycholesterol promotes cell-autonomous, ER-positive breast cancer growth. Cell Rep. 5:637–45.

van der Wulp MYM, Verkade HJ, Groen AK. 2013. Regulation of cholesterol homeostasis. Mol. Cell. Endocrinol. 368:1–16.

Yu X, Narayanan S, Vazquez A, Carpizo DR. 2014 Apr 23. Small molecule compounds targeting the p53 pathway: are we finally making progress? Apoptosis.

Zheng L, Ren JQ, Li H, Kong ZL, Zhu HG. 2004. Downregulation of wild-type p53 protein by HER-2/neu mediated PI3K pathway activation in human breast cancer cells: its effect on cell proliferation and implication for therapy. Cell Res. 14:497–506.

Zhou CK, Check DP, Lortet-Tieulent J, Laversanne M, Jemal A, Ferlay J, Bray F, Cook MB, Devesa SS. 2016. Prostate cancer incidence in 43 populations worldwide: An analysis of time trends overall and by age group. Int. J. Cancer 138:1388–400.