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The Role Of The Cholesterol Metabolite, 27-Hydroxycholesterol, In Colon Cancer Cells

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THE ROLE OF THE CHOLESTROL METABOLITE, 27-HYDROXYCHOLESTROL, IN COLON CANCER CELLS

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Jessica Warns
August, 2018

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ABSTRACT

Colorectal Cancer (CRC) is a major health concern with more than 600,000 deaths worldwide each year. Several risk factors for CRC exist including a diet rich in fat and cholesterol. However, the link between cholesterol and CRC is controversial. It is possible that oxysterols, which are active cholesterol metabolites, may correlate better with CRC than cholesterol itself. The most abundant oxysterol in the plasma is 27-hydroxycholesterol (27-OHC), which is a ligand for the nuclear receptors estrogen receptor (ER) and liver-x-receptor (LXR). 27-OHC has been shown to be involved in breast and prostate cancers, but its role in colon cancer remains unexplored. Therefore, we sought to determine the role of 27-OHC in colon cancer cell lines. We also determined the impact of 27-OHC on cellular differentiation through measurement of the protein SLFN12. Using two colon cancer cell lines (Caco2 and SW620), an MTT assay and CyQUANT® proliferation assays, an LDH cytotoxicity assay, a TUNEL assay, western blotting, immunofluorescence, and a scratch assay, we determined 27-OHC's effects on cellular proliferation, migration, cytotoxicity, and apoptosis. We also determined the role of ER and LXR in cellular proliferation. We found that treatment of Caco2 and SW620 cells with 27-OHC induced a decrease in cell proliferation without a detectable amount of cellular cytotoxicity or apoptosis. Additionally, we found that the observed decrease in cell proliferation is independent of ER and LXR, as 27-OHC did not activate the LXR target genes ABCG1 and ABCA1 nor lead to the nuclear localization of

ER. Additionally, we found that 27-OHC leads to a decrease in the phosphorylated and activated form of AKT, a molecule involved in cell cycle progression, protein synthesis, and cellular survival. We also found that 27-OHC increased cellular migration through a scratch assay. 27-OHC decreased the epithelial marker E-cadherin without changes to the mesenchymal markers N-cadherin. In order for cells to migrate, they need to stop proliferating. In conclusion, our data shows that 27-OHC treatment leads to an increase in cellular migration through a loss of proliferation.

CHAPTER I

INTRODUCTION

Cancer

Cancer is a collection of diseases in which the growth and division of cells becomes uncontrolled. According to Hanahan and Weinberg, cancer cells are characterized by sustained proliferation signaling, evasion of growth suppression, resistance to cell death, enabling of replicative immortality, induction of angiogenesis, and activation of invasion and metastasis (Hanahan and Weinberg, 2011). Cancer cells are often characterized by aneuploidy or having an abnormal number of chromosomes, leading to genetic instability (Chial, 2008). Cancer cells also lack differentiation, meaning that they are not specialized cells. Cancer cells also go through tumor metastasis or migration during which cancer cells from the primary tumor move to another part of the body to form a secondary tumor.

Cancer relies on protooncogenes to drive cellular proliferation. Normally, proto-oncogenes promote cell division and inhibit cell differentiation and cell death. However, when a protooncogene is mutated, it is known as an oncogene, which can be constitutively active, leading cells to 'out of control' proliferation and cancer. Examples of proto-oncogenes include RAS, WNT, MYC, and TRK. Activation can occur through mutations that lead to "hyperactive" gene products, mutations in the promoter region that increase transcription, gene amplification, and chromosomal translocation that leads to increased transcription or to a fusion protein with oncogenic potential (Chial, 2008).

Tumor suppressor genes are genes that normally act as a check to cell division when a cell undergoes DNA mismatch repair or apoptosis if the DNA damage is too severe. When there is a mutation in a tumor suppressor gene, the cell cannot go through these checks, which leads to proliferation. It follows the two-hit hypothesis in which two genetic events that lead to a loss of a tumor suppressor need to occur before uncontrolled cell proliferation takes place. Examples include Rb and p53 (Chial, 2008).

A major controller of cell proliferation is apoptosis. Apoptosis occurs during development, wound healing, and aging. Apoptosis or programed cell death has two main pathways, the extrinsic and intrinsic pathways, which are connected and merge at the execution phase or the last part of apoptosis. Apoptosis involves proteolytic caspases, which are a group of enzymes that cleave proteins at aspartic acid residues. Several caspases have been discovered that are organized into initiators (caspase 2,8,9,10), effectors (caspase 3,6,7), and inflammatory caspases (1,4,5)(Elmore, 2007). Another prominent protein involved in apoptosis is annexin V, a phosphatidylserine-binding protein. Phosphatidylserine is found on the cytosolic part of the membrane. However, during apoptosis, scramblase allows for the movement of phosphatidylserine from the cytoplasmic side to the extracellular side, signaling apoptosis. Apoptosis then can be assessed through the measurement of Annexin V (Verhoven, 1995). Positive controls for apoptosis include doxorubicin, 5-Florouracil, Paclitaxel, Vinblastine, and staurosporine.

The extrinsic pathway involves the death transmembrane receptors, also known as the death receptor pathway. The death receptors are part of the tumor necrosis factor (TNF) superfamily that have an extracellular, cysteine-rich domain and an intracellular death domain, which is responsible for bringing signals into the cell. Some ligands for the

death receptor include FASL, TNF-α/TNFR1(Suliman et al., 2001), and Apo3L and Apo2L (Elmore, 2007). Once a ligand binds, cytoplasmic adapter proteins interact with the death receptor. Next, a death-inducing signaling complex (DISC) is formed, causing activation of procaspase-8 and leading to the execution phase (see below)(Elmore, 2007).

Another pathway is the intrinsic or mitochondrial pathway in which non-receptor stimuli lead to intracellular signaling events. Some stimuli include radiation, toxins, and free radicals. The stimuli alter the mitochondrial membrane, resulting in the release of cytochrome c, which binds to Apaf-1 and procaspase 9 to form an apoptosome (Hill et al., 2004). Later during the apoptotic process, AIF and endonuclease G are released and then enter the nucleus, resulting in DNA fragmentation (Li et al., 2001).

The Bcl-2 family of proteins control apoptosis by regulating mitochondrial membrane permeability. The dephosphorylated form of Bad, a member of the Bcl-2 family, releases cytochrome c from the mitochondria (Zha et al., 1996). Additionally, Bad can promote cell death by counteracting Bcl's effects, which normally promote cellular death (Yang et al., 1995). p53 can regulate apoptosis through Puma and Noxa, leading to upregulation of capase-9 (Oda et al., 2000).

The execution phase involves cleavage of caspase-3, DNA fragmentation, degradation of cytoskeletal and nuclear proteins, formation of apoptotic bodies, and uptake through phagocytosis. The execution pathway activates cytoplasmic endonucleases and proteases that degrade nuclear and cytoskeletal proteins. Additionally, caspase 3 is activated by the initiator caspases (8,9, and 10). Caspase-3 activates an endonuclease and cytoskeletal rearrangement. Phagocytosis of the apoptotic cell is the

last step. Caspase-3 and 8 are involved in the movement of phosphotidylserine to the outer leaflet, signaling phagocytosis without an inflammatory response.

Cancer cells can become resistant to apoptosis through overexpression of anti-apoptotic factors and downregulation of pro-apoptotic factors such as Bcl-2 and Bax, respectively (Elmore, 2007). In addition to the regulation of certain proteins, cancer cells can prevent the phagocytosis of immune cells. This can occur through downregulation of the death receptor response pathway involving FasL and alternations in p53 signaling through upregulation of AKT (Cheng et al., 1994; Wang and Harris, 1997). Other diseases that require apoptosis include HIV/AIDS (Li et al., 1995), Alzheimer's disease (Ethell and Buhler, 2003), and ischemia (Freude et al., 2000).

Another important aspect of cancer progression is tumor migration from the area of the primary tumor to a secondary area in the body. Tumor migration is the most common cause of cancer-related deaths (Gupta and Massagué, 2006; Steeg, 2006). Migration involves the movement of cells through the ECM with the maintenance of cell-cell junctions. Migration can be divided into amoeboid movement and mesenchymal movements. Amoeboid movement involves cells moving as rounded bodies without the involvement of cell attachment or through actin-rich filopodia outgrowths. Mesenchymal migration involves strong attachment to the ECM. Cells that complete this type of migration include fibroblasts, sarcomas, and tumor cells that undergo a major event in tumor migration, epithelial to mesenchymal transition (Kramer et al., 2013). Epithelial to mesenchymal transition (EMT), which results in the attainment of an invasive mesenchymal phenotype, is regulated by several transcription factors including SNAI1, SNAI2, ZEB1, and TWIST1. These transcription factors work to repress epithelial genes

such as E-cadherin and lead to an upregulation of mesenchymal markers such as N-cadherin or Vimentin. In order for the mesenchymal cells to attach to a secondary location, they need to undergo a mesenchymal to epithelial transition (MET) (Warns et al., 2016).

Cancer can be classified according to the type of cells in which the cancer originates. Examples include carcinoma, which is a cancer of epithelial cells, sarcoma or cancer of connective tissues, lymphoma and leukemia from blood, and germ cell that arises from pluripotent cells. Several types of carcinomas exist including basal cell, squamous, and adenocarcinoma. Adenocarcinomas are tumors that arise from glandular structures and include breast and colon cancers (Cooper, 1992).

Colorectal Cancer

Colorectal Cancer (CRC) is a cancer of the large intestines and/or the rectum. The colon is a glandular structure that contains a mucosa layer. It functions in taking in water and nutrients along with packing waste material into feces to exit the body. There are four parts of the large intestine: the ascending colon, transverse colon, descending colon, and sigmoid colon. There is a vast amount of cellular turnover in the colon, which increases the chance of developing cancer.

CRC is a major health concern. In the United States, CRC is the third most diagnosed cancer, affecting 1 out of approximately 22 people in their lifetime. It is estimated that 135,000 people were diagnosed and more than 50,000 died from CRC in the United States in 2017 (American Cancer Society). Globally, it is the third most common type of cancer, leading to an estimated 1.4 million patients being diagnosed in

2016 (Verma and Kumar, 2017) with more than 600,000 deaths (Dolatkhah et al., 2015; Verma and Kumar, 2017).

There are two main models of colon cancer progression, the adenoma-carcinoma sequence and inherited types (Figure 1) (Smith et al., 2002). A total of 95 percent of CRC cases arise from non-cancerous growths or polyps (adenomas) of the mucosal lining of the colon that develop into cancerous adenocarcinomas or tumors that arise from glandular structures. This type of cancer progression ranges from years to even a decade or more. If the polyp progresses to cancer, it becomes an in situ tumor localized in the outer layer of the colon. The tumor then begins to grow through the different layers of the colon, including the colon wall. The tumor then becomes regional and grows outside the colon wall to nearby lymph nodes. Finally, if left untreated, the tumor becomes distant or metastasized (i.e., the cancer spreads to other parts of the body). The most common sites for metastasis include the liver or the lungs (Chirica et al., 2012). On the molecular level, the adenoma-carcinoma sequence is a multistep sequence of molecular events that contains three pathways including chromosomal instability (CIN), microsatellite instability (MSI), and CpG island methylator phenotype (CIMP).

The first step in the adenoma-carcinoma sequence leading to chromosomal instability is acquiring mutations in the adenomatous polyposis coli (APC) gene, which is a tumor suppressor gene. APC is normally part of the Wnt/β-catenin signaling pathway. Normally, APC inhibits β-catenin and leads to upregulation of Wnt signaling. When APC is mutated, there is an increase in β-catenin and a downregulation of Wnt signaling

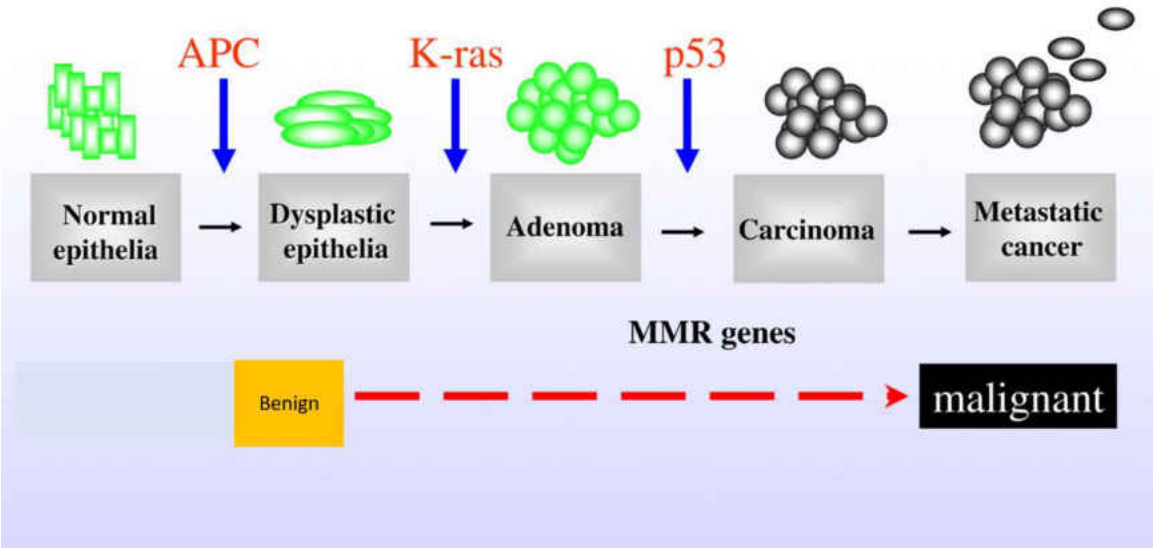

Figure 1. The Adenocarcinoma sequence. A model of the order and timing of genetic changes during the adenocarcinoma sequence. Reproduced with permission from Smith et al., 2002. Copyright (2002) National Academy of Sciences, U.S.A.

leading to changes in the cytoskeleton, proliferation, and cellular migration (Nelson et al., 2012). Additionally, mutations in APC can lead to upregulation of c-myc and cyclin D1, controlling the cell cycle (see above).

The next step in the adeno-carcinoma sequence involves acquiring chromosomal instability in KRAS, a small GTPase involved in the G-protein signal transduction pathway. Mutations in KRAS are resistant to EGFR signaling. Normanno et al. showed that approximately 40 percent of CRC patients have a KRAS mutation, which is often located in codons 12 and 13. (Allegra et al., 2009; Normanno et al., 2009). KRAS is a part of the MAPK pathway, and it is involved in cell proliferation signaling pathways including MAPK, C-Jun, and MAPK14 (Fang and Richardson, 2005; Hommes et al., 2003).

After mutation of KRAS, mutations develop in BRAF, a serine/threonine-specific protein kinase and oncogene. These mutations are part of RAS/MAPK pathway that controls cell proliferation, differentiation, migration, and apoptosis. This pathway is activated when signaling molecules from the plasma membrane activate RAS proteins, which then recruit the RAF kinase family to the plasma membrane and then signal to MEK and then ERK. A common mutation in which a valine is substituted by glutamic acid occurs on codon 600 and leads to a constitutively active form, resulting in activation of the MAPK pathway. These patients have a poor prognosis and are resistant to treatment (Samowitz et al., 2005). This mutation is found in five to fifteen percent of CRC patients (Davies et al., 2002; Day et al., 2015; Pakneshan et al., 2013).

Another common mutation in colon cancer that leads to chromosomal instability occurs in p53, a tumor suppressor. Normally, p53 levels are low, but it is activated by genomic damage. p53 is controlled by MDM2, an E3 ubiquitin protein ligase. When there is DNA damage, p53 is no longer degraded, resulting in cell-cycle arrest and apoptosis. However, mutation of p53 results in dysregulation of the cell cycle, allowing for cell proliferation. p53 leads to activation of genes such as p21, Gadd45, Bax, and NoxA (Blagosklonny, 2002).

Microsatellite instability (MSI) is another pathway that leads to genomic instability. Microsatellites are sequences of nucleotide repeats ranging from one to six base pairs. These repeats are susceptible to mutations such as insertions and deletions, leading to frameshift mutations. MSI is caused by silencing of mismatch repair genes (MMR), specifically through DNA hypermethylation of the MLH1 promoter. This is

characteristic of the HNPCC type of colon cancer and also fifteen percent of sporadic CRC cases (Bogaert and Prenen, 2014; 2004).

The last major pathway involved in CRC is the CpG island methylation pathway (CIMP). This is an epigenetic mechanism of DNA methylation at CG dinucleotide sequences or CpG. DNA methylation adds a methyl group through a DNA methyltransferase (DNMT) to 5' cytosine to form 5-methylcytosine. CpG islands are sections of CpG rich regions, and when there is DNA methylation at promoter CpG islands, there is often gene silencing (Nazemalhosseini Mojarad et al., 2013; van Rijnsoever et al., 2002). In cancer cells, some CpG islands are unmethylated while some are hypermethylated, resulting in gene silencing (Hughes et al., 2012; Toyota et al., 1999). Cancer with a higher amount of CpG methylation is known as the CpG island methylator phenotype of CIMP. In CRC, CIMP leads to inactivation of tumor suppressor genes and mismatch repair genes, leading to tumorigenesis.

There are two types of hereditary colorectal cancer, hereditary nonpolyposis colorectal cancer (HNPCC) and familial adenomatous polyposis (FAP). HNPCC, also known as Lynch syndrome, is an autosomal dominant genetic disorder that increases a patient's risk of developing colon cancer along with other cancers including endometrial, ovarian, stomach, and skin cancers. HNPCC accounts for 15 percent of CRC cases and results in more than 80 percent progressing to cancer. Specifically, HNPCC is caused by germline mutations in mismatch repair (MMR) genes such as hMSH2 and hMLH1 in which one copy of the mutant MMR gene is inherited and then the second allele is mutated in an early event during cancer progression. Since these cells no longer contain alleles for the MMR genes, there is an increase in mutations that leads to tumorigenesis.

Genetic testing for mutated MMR genes and also microsatellite instability testing are used for screening (Jäger et al., 2001).

FAP is another genetic cause of CRC. It is present in 1 in 22,000 people and represents less than one percent of CRC cases. FAP is an autosomal dominant genetic disorder in which 100's to 1000's of adenomatous polyps develop and later become cancerous. The polyps usually begin to develop during the teenage years with 100 percent of patients developing CRC usually by the age of 40. Most patients develop polyps in the stomach and small intestines as well. FAP develops from mutations in tumor suppressor genes such as APC gene through an autosomal dominant inheritance (Boland et al., 2018).

CRC risk factors.

Several risk factors exist for CRC. One risk factor is age with approximately 93 percent of cases occurring in those over the age of 50 (Gandhi et al., 2017). African Americans have the highest colorectal cancer incidence of all racial groups in the United States (Jemal et al., 2008; Robbins et al., 2012; Wilkins et al., 2012). The risk of CRC is also dependent on whether a family history of CRC exists. The risk of being diagnosed with CRC is between 1.9 and 4.4 times higher in those with a family history of CRC compared to those with no family history (Henrikson et al., 2015). Additionally, another risk factor is having inflammatory bowel disease such as Crohn's disease (Jewel Samadder et al., 2017).

Studies have shown that alcohol consumers, specifically moderate drinkers, are 3 times more likely to develop CRC (Cope et al., 1991) with men being four times more likely (Sandler et al., 1993) to develop polyps that may become cancerous. Fish, fiber,

and milk consumption decrease CRC risk by 12, 25, and 26 percent, respectively (Baena and Salinas, 2015), while consumption of red and processed meat is associated with an increased risk of CRC (Zhao et al., 2017). In a meta analysis study, current smokers had an increased risk of CRC that was related to both the duration and amount of cigarettes smoked (Liang et al., 2009). Also, in an analysis of 106 observational studies, it was found that increasing the amount of cigarettes smoked in a dose response significantly increased one's risk of developing colorectal cancer (Botteri et al., 2008). In a meta analysis of 30 prospective studies, an increase in body mass index (BMI) of five units increased the risk of colorectal cancer in both genders (Larsson and Wolk, 2007). A meta analysis of thirty-one studies found a link between obesity and colorectal cancer (Moghaddam et al., 2007). Another meta analysis of twenty-one studies found that the risk of developing CRC was 27 percent lower among physically active people relative to those with a sedimentary lifestyle (Boyle et al., 2012; Wolin et al., 2011).

Cholesterol has been considered to be a risk factor for cancer, but this is controversial. Several epidemiological studies suggested that higher cholesterol levels were associated with risk of cancer development (Shafique et al., 2012). Other studies have shown that lowering cholesterol levels with statins reduced the risk of some cancers (Jacobs et al., 2011). Specifically in CRC, an observational study found a decreased risk of CRC with statin therapy (Cardwell et al., 2014; Liu et al., 2014). However, Mamtani et al. found that those with lower cholesterol levels had an increased risk of CRC (Mamtani et al., 2016).

Cholesterol

Cholesterol is a four-ring steroid molecule with a single polar group at the 3b position, forming a 3b-hydroxyl moiety. Cholesterol is synthesized either de novo or through dietary supplementation. De novo cholesterol synthesis from acetyl CoA occurs through the mevalonate pathway. Cholesterol levels are regulated through sterol regulatory element-binding proteins (SREBPs). When cholesterol or steroid levels are low in the ER, SREBP is activated to produce more. When sterol levels are low, SREBP is trafficked to the Golgi from the ER through complex SREBP cleavage activating protein (SCAP) and leads to the production of fragments that enter the nucleus, activating cholesterol production genes and those involved with the LDL receptor to activate genes involved in cholesterol uptake and synthesis (Figure 2).

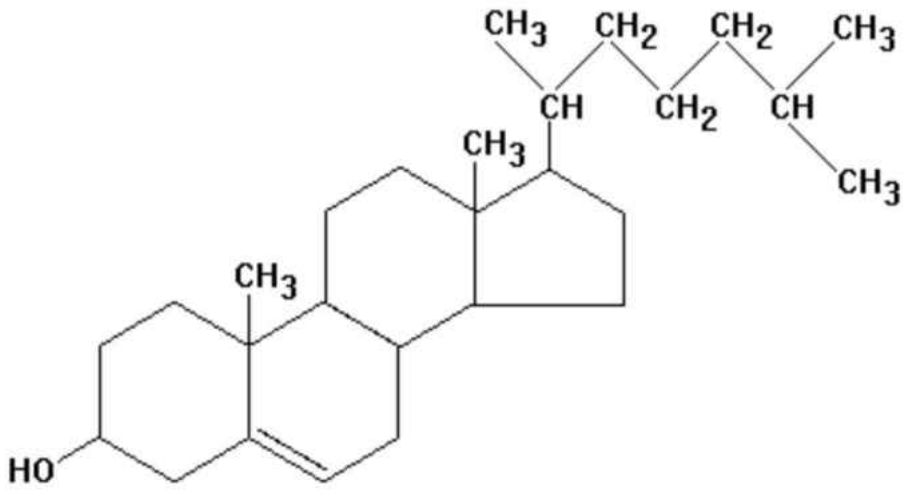

Figure 2. Cholesterol structure. The structure of cholesterol with four rings structure.

Cholesterol obtained by consuming animal products is transferred from the liver to the rest of the body. To assist in the long distance travel of cholesterol and due to its hydrophobicity, cholesterol is first esterified and packed along with triglycerides into lipoproteins called chylomicrons. At the same time, hepatocytes secrete very low-density lipoproteins that are processed to low density lipoprotein, allowing for cholesterol to be transported to other cells through endocytosis and further processed into cholesterol through the lysosome. Specifically, LDL that contains packaged cholesterol binds to the LDL receptor on the plasma membrane, which is endocytosed and binds to an early endosome and later a lysosome where cholesterol is released from the LDL. The LDL receptor is then trafficked back to the plasma membrane. When cholesterol is highly abundant, it binds to high-density lipoproteins (HDL), returns to the liver, enters the small intestines through secretion of bile, and is removed through the digestive tract. This process is regulated through liver-x-receptor (LXR), which regulates the ABC transporters that export cholesterol from cells. Two ABC transporters exist, ABC transporter A1 (ABCA1) and ABCG1(Ikonen, 2008).

Cholesterol is found in the plasma membrane where it helps to create a semipermeable membrane controlling fluidity and the permeability of small molecules and regulating membrane trafficking through interaction with lipids and proteins (Berkowitz, 2009; Maxfield and Tabas, 2005; Róg et al., 2009). Cholesterol along with sphingolipids can be concentrated to specific parts of the plasma membrane, forming lipid rafts (van Meer and Simons, 1988; Simons and Sampaio, 2011). One type of lipid raft is a caveolae or small invaginations of the plasma membrane. Cholesterol, through caveolae, can influence extracellular signals such as G-protein coupled receptors

(GPCR). For example, cholesterol from the cell surface can regulate GPCR signaling through interaction with the transmembrane domain (7TMD) (Luchetti et al., 2016) and by bringing the GPCR closer to second messengers (Incardona and Eaton, 2000). It is also involved in Hedgehog signaling by activating Smoothened (SMO) through its extracellular cysteine-rich domain (Luchetti et al., 2016).

Statins (drugs used to lower cholesterol) are competitive inhibitors of the enzyme HMG-CoA reductase, the rate-limiting step of cholesterol synthesis that converts HMG-CoA to mevalonate. Activation of the mevalonate pathway influences tumor growth, reduces apoptosis, affects cellular survival (Li et al., 2006; Pommier et al., 2010), alters cellular adhesion (Murai, 2012), and leads to a poor prognosis (Clendening et al., 2010) (Figure 3).

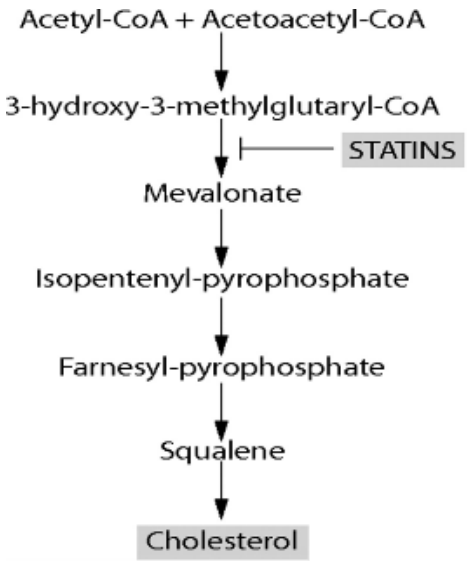

Figure 3. Cholesterol biosynthesis pathway. The cholesterol pathway schematic with the location of the mode of action of statins.

Oxysterols

Cholesterol is a precursor to several important molecules such as bile acids, steroid hormones, vitamin D, and oxysterols. Oxysterols are cholesterol oxidation products with the addition of a polar group such as hydroxyl, keto, or epoxy groups. This addition can occur either nonenzymatically through autoxidation or diet, or enzymatically. Nonenzymic reactions usually result in oxidation on the steroid ring structure, while enzymatic reactions usually result in oxidation products on the side chains. Nonenzymatic reactions occur through reactive oxygen species (ROS) found in the cell (Brown and Jessup, 2009; Kulig et al., 2016) (Figure 4) (Olkkonen et al., 2012). The enzymes involved in the production of oxysterols are oxidoreductases (cytochrome P450), hydrolases, and transferases. For example, CYP27A1, similar to P450, is found in the liver and synthesizes 27-hydroxycholesterol (Mutemberezi et al., 2016). Additionally, oxysterols can be obtained through animal products such as meats and dairy. Once ingested, oxysterols are esterified and incorporated into chylomicrons and eventually LDL, which can then transfer oxysterols into cells (see above) (Staprans et al., 2003, 2005).

Oxysterols are ligands for the liver x receptor [LXR, a nuclear receptor that has a variety of functions (see below)], RAR-related orphan receptor, and the estrogen receptor. Oxysterols are also known to bind to members of G-protein coupled receptors (GPCRs) such as GPR183, (Hannedouche et al., 2011; Liu et al., 2011), GPR17(Sensi et al., 2014), and CXCR2 (Raccosta et al., 2013). In addition to GPCRs, oxysterols can also bind to and regulate several proteins including insulin-induced gene protein (INSIG)

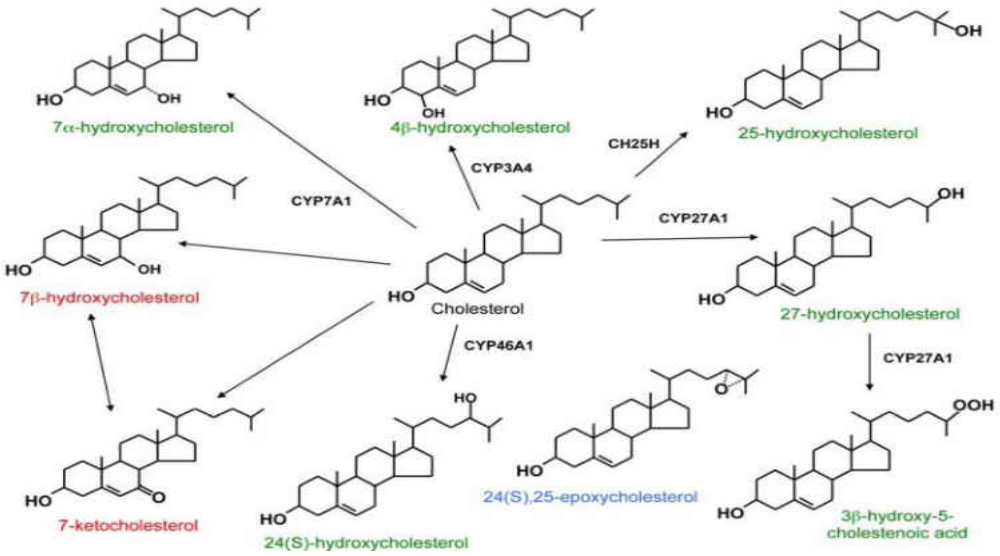

Figure 4. Oxysterols structures. The enzymatically derived species are indicated with green, products of cholesterol autoxidation with red, and a species derived from a shunt of the cholesterol biosynthetic process with blue print. Reproduced with permission from Olkkonen et al., 2012.

(Radhakrishnan et al., 2007), sterol regulatory element-binding protein, Niemann-Pick protein (NPC) (Infante et al., 2008), and the steroidogenic acute regulatory-related lipid transfer (START) (Calderon-Dominguez et al., 2014) and oxysterol-binding protein (OSBP) families (Olkkonen and Li, 2013).

Oxysterols can modify the plasma membrane, eliciting changes in permeability and signal transduction and thereby altering Hedgehog signaling, for example. In addition, changes in the plasma membrane can also lead to cellular cytotoxicity, which can lead to apoptosis (Aupeix et al., 1995; Salvayre et al., 2002). Oxysterols have been shown to lead to cytotoxicity in cell culture (Dasari et al., 2010; Meynier et al., 2005; Vejux et al., 2008; Wielkoszyński et al., 2006). However, the effects of oxysterols on apoptosis depend on the type of oxysterol.

Cholesterol metabolism is controlled by the transcription factors of sterol regulatory element-binding proteins (SREBPs). Specifically, the maturation of SREBPs is regulated by SREBP cleavage-activating protein (SCAP), which binds with SREBP at the ER. When cholesterol is low, the SREBP-SCAP complex enters the Golgi where it is processed to a leucine zipper transcription factor that transports to the nucleus and binds sterol regulatory elements (SRE). When cholesterol levels begin to increase, SCAP interacts with insulin-induced gene (INSIG), preventing the SREBP-SCAP interaction in the ER. Oxysterols can regulate cholesterol synthesis. Oxysterols and cholesterol inhibit the transport of SREBP to the Golgi. They also interact with INSIG and oxysterol that then binds with SCAP.

Oxysterols have been shown to be involved in both innate and adaptive immunity. Oxysterols increase the levels of inflammatory cytokines and chemokines such as TNF-α, Il-1β, and Il-8. Oxysterols associate with the innate immune system through interferon, leading to a transcriptional increase in 25-hydroxylase, which leads to an increase in 25-hydroxycholesterol (Park and Scott, 2010). Inhibition of 25-hydroxylase in macrophages also increases IL-1 via SREBP downregulation (Reboldi et al., 2014). 25-hydroxycholesterol regulates B cell proliferation and IgA levels (Bauman et al., 2009). 27-hydroxycholesterol has also been shown to inhibit viral infections (Civra et al., 2015).

Oxysterols are also involved in several diseases. 27-hydroxycholesterol can induce atherosclerosis (Umetani et al., 2014). Additionally, oxysterols are involved in Alzheimer's disease (Lütjohann et al., 2000; Mateos et al., 2011), Huntington's disease (Leoni et al., 2013), and cancer. Oxysterols play a role in cancer cell proliferation both

through proproliferative and antiproliferative effects depending on the oxysterol and the type of cancer. Little is known about their effects in CRC, but they have been shown to play an antiproliferative role via LXR (Roussi et al., 2005). Oxysterols also regulate TGF-β1 signaling, leading to proliferation of colon cancer cells.

27-Hydroxycholesterol

The most abundant oxysterol in the plasma is 27-hydroxycholesterol (27-OHC) (Duane and Javitt, 1999), which is made in the mitochondria. The physiological concentration of 27-OHC ranges from between 0.15–0.73μM but can reach millimolar ranges under some physiological conditions (Brown and Jessup, 1999). 27-OHC is synthesized by the mitochondrial enzyme CYP27A1 and hydroxylated by the endoplasmic reticulum protein CYP7B1 to form a 7α-hydroxylated metabolite, which forms bile acids (Russell, 2000, 2009).

27-OHC functions to maintain proper cholesterol efflux from peripheral tissues. For example, patients with mutated CYP27A1 exhibit excess amounts of cholesterol in peripheral macrophages (Björkhem and Leitersdorf, 2000). 27-OHC has been shown to suppress lipid accumulation in 3T3-L1 cells (Shirouchi et al., 2017). 27-OHC is a macrophage-synthesized cholesterol metabolite that can enhance inflammation in macrophages, which are detected in abundance in atheromatous lesions (Kim et al., 2013a). 27-OHC is a ligand for liver-X-receptor (LXR), a nuclear receptor that has a variety of functions (Fu et al., 2001). 27-OHC has also been shown to act as a selective estrogen receptor modulator (SERM) (DuSell et al., 2008; Umetani and Shaul, 2011; Umetani et al., 2007). 27-OHC is also a ligand for steroidogenic factor 1 (SF-1), a

member of the orphan nuclear receptor superfamily expressed in the adrenal and gonadal glands. 27-OHC binds to and upregulates SF-1 transcription (Lala et al., 1997).

27-OHC plays a role in several cancers including breast (Nelson et al., 2013), endometrial (Gibson et al., 2018), prostate (Raza et al., 2016, 2017), melanoma (Tian et al., 2018), and lung cancer (Hashimoto et al., 2016). Specifically, 27-OHC has been shown to reduce AKT phosphorylation and activation in prostate cancer cell lines (Pommier et al., 2010). 27-OHC also regulates the Janus Kinase (JAK)/signal transducer and activator of transcription (STAT) signaling pathway via LXR (see below) (Mateos et al., 2011) and LXR-independent pathways (Romeo and Kazlauskas, 2008). 27-OHC has been shown to create vascularization by increasing the expression of vascular endothelial growth factor alpha (VEGFα) (Zhu et al., 2016) and to induce hypoxia inducible factor 1 alpha (HIFα) (Krock et al., 2011). Additionally, 27-OHC can facilitate the migration of tumor cells from one area to another (Shen et al., 2017). Furthermore, Raza et al. showed that 27-OHC reduces the amount of cellular invasion in prostate LNCaP and PC3 cell lines via estrogen receptor β. 27-OHC was also shown to upregulate connective tissue growth factor (CTGF), Patched (Ptc), and Gil family transcription factors Gil2 and Gil3, proteins that are positively correlated with metastasis in PC3 cell lines (Raza et al., 2017).

27-OHC is involved in atherosclerosis (Umetani et al., 2014), Alzheimer's disease (Marwarha et al., 2012), Parkinson's disease (Marwarha et al., 2011), and inflammation (Kim et al., 2013a). Specifically in breast cancer, 27-OHC has been shown to reduce chemotherapy drug doxorubicin in MCF7 breast cancer cells through estrogen and mTOR signaling (Wang et al., 2017). 27-OHC leads to stabilization of c-myc through downregulation of myc transcriptional regulators including PP2A, SCP1, and FBW7 (Ma

et al., 2016) in MCF-7 breast cancer cells. 27-OHC also leads to a decrease in E-cadherin and therefore induces a mesenchymal phenotype in MCF-7 cells (Sierralta, 2011). Additionally, 27-OHC induces EMT through increased expression of matrix metalloproteinase 9 (MMP9) and STAT3 in both estrogen receptor positive and negative breast cancer cell lines (Shen et al., 2017). 27-OHC increases the number of polymorphonuclear neutrophils (PMNs) and decreases the number in cytotoxic CD8+ T-cells (Baek et al., 2017).

27-OHC plays several roles in neurodegenerative diseases. A risk factor for Alzheimer's disease (AD) is dysregulation of cholesterol homeostasis (Famer et al., 2007). In astrocyte cells (C6 cell line), 27-OHC increases the levels of reactive oxygen species and downregulates Nrf2 expression, suggesting that 27-OHC leads to oxidative damage or stress via Nrf2 signaling (Ma et al., 2015). 27-OHC has been shown to accumulate in the brains of AD patients including those carrying the Swedish amyloid precursor protein (APPswe) mutation (Shafaati et al., 2011). 27-OHC also increases Aβ peptide production and increases the levels of ER stress-specific markers including the leucine zipper transcription factor CHOP in ARPE-19 pigmented retinal epithelium cells (Dasari et al., 2010). Activated CHOP can generate reactive oxygen species and increase Aβ levels, both of which are hallmarks of AD (Prasanthi et al., 2011). Additionally, our lab has shown that the adipocytokine leptin is inhibited by 27-OHC through activation of ER stress through CHOP (Marwarha et al., 2012). 27-OHC has been shown to decrease tyrosine hydroxylase (TH) and increase α-synucelin accumulation in SH-SY5Y cells through the estrogen receptor and Liver-X-receptor (LXR) (see below) in a Parkinson's disease model (Marwarha et al., 2011).

27-OHC also plays a role in inflammation. In monocytic cells, treatment with 27-OHC resulted in increased synthesis and secretion of the cytokine TNF-α, influencing atherosclerosis (Kim et al., 2013a). In the Herpes Simplex-1 virus (HSV-1), 27-OHC leads to antiviral activity through activation of IL-6 and NF- κB (Cagno et al., 2017). 27-OHC also leads to antiviral activity to human papillomavirus-16 (HPV-16), human rotavirus (HRoV), and human rhinovirus HRhV viruses (Civra et al., 2015). 27-OHC and other oxysterols decrease CD36 and CD204 surface receptor expression, reduce reactive oxygen species, and increase secretion of IL-10 during monocyte differentiation to macrophages (Marengo et al., 2016). In macrophages and aortic smooth muscle cells (HASMC), 27-OHC leads to an increase in lysosomal-independent cell death (Riendeau and Garenc, 2009). In THP-1 monocytic cells, 27-OHC induces secretion of CCL2 and results in AKT-dependent migration of these cells (Kim et al., 2013b).

Liver-X-Receptor

In addition to 27-OHC acting as a ligand for the estrogen receptor, 27-OHC also acts as a ligand for another group of nuclear receptors, the liver-x-receptors. Two isoforms of LXR exist, LXRα (NR1H3) and LXRβ (NR1H2). Human LXRα and LXRβ share 80% of their amino acid sequences. LXR contains a DNA-binding domain and a ligand-binding domain. LXRα is found in the liver, intestines, and adipose tissue, while LXRβ is found to be expressed in almost all tissues (Hong and Tontonoz, 2014). In addition to 27-OHC, other molecules also act as ligands for LXR including other oxysterols such as 22-hydroxycholesterol, 20-hydroxycholesterol, and 24-hydroxycholeterol (Janowski et al., 1999), as well as phytosterols (Plat et al., 2005)

and bile acids (Song et al., 2000). Synthetic ligands for LXR exist, including the agonist TO901317 (Schultz et al., 2000) and the agonist GW3965 (Collins et al., 2002).

LXR binds to DNA at LXR-response elements (LXREs) as a heterodimer with the retinoid X receptor, leading to activation of several genes including fatty acid synthase (Joseph et al., 2002), phospholipid transfer protein gene (Mak et al., 2002), sterol regulatory element-binding protein 1c gene (Repa, 2000), and ATP-binding cassette transporter G1 (ABCG1) (Kennedy et al., 2001). Without a ligand, LXR-RXR is bound to the DNA but with co-repressors (Hu et al., 2003; Svensson, 2003). With a ligand, a conformational change in LXR-RXR occurs, leading to the removal of co-repressors and a gain of co-activators such as E1A-associated protein p300 (EP300) (Huuskonen et al., 2004) and activating signal co-integrator 2 (ASC2) (Kim et al., 2009).

LXRs act as "cholesterol sensors" by which oxysterols accumulate when cholesterol is high. When this happens, LXR induces activation of genes involved in reverse cholesterol transport. Such genes include those involved in bile acid synthesis, cholesterol absorption, and cholesterol excretion. Specifically, high cholesterol levels signal via oxysterols to trigger bile acid synthesis in which LXR binds to the LXRE on CYP7A1 and synthesize 7α-hydroxycholesterol, eventually leading to the production of bile acids (Peet et al., 1998). LXR also regulates bile acid production by upregulating UGT1A3, an enzyme that produces bile acids (Zhao and Dahlman-Wright, 2010). Free cholesterol can also be excreted into bile acids from the liver through ABCG5 or ABCG8 (Yu et al., 2002). LXRs also regulate reverse cholesterol transport, a process in which excess cholesterol in tissue is returned to the liver through HDL and then made into bile

through activation of ABCA1, ABCG2, and apolipoprotein E (Laffitte et al., 2001) as well as through cholesterol efflux.

In addition to playing a role in cholesterol homeostasis, LXRs also play a role in metabolism, specifically lipogenesis and glucose metabolism. For example, mice that have a mutated LXRα gene exhibit downregulation of the genes involved in fatty acid metabolism including sterol regulatory element-binding protein (SREBP-1c) (Repa, 2000), fatty acid synthase (FAS) (Joseph et al., 2002), and acetyl CoA carboxylase (ACC) (Peet et al., 1998). Activating LXRs in mice increases triglycerides in the plasma, and treatment with LXR agonists decreases hyperglycemia (Cao et al., 2003; Mitro et al., 2007).

LXR receptors have been shown to be involved in several diseases including atherosclerosis and cancers such as breast, prostate, and colon cancer. Atherosclerosis forms when macrophages uptake oxysterols, which eventually leads to foam cell formation. LXR agonists can lead to a decrease in atherosclerosis (Bischoff et al., 2010). In breast cancer for example, LXR inhibits cellular proliferation through G1 arrest via an estrogen-dependent molecule known as estrogen sulfotransferase and estrogen-independent molecules (Gong et al., 2007; Vedin et al., 2009). In prostate LNCaP cells, activation of LXRs leads to a decrease in cellular proliferation by decreasing cells in S-phase and a decrease in xenografts in nude mice. Additionally, activation of LXRs through TO901317-activated apoptosis downregulates AKT signaling and cytokine signaling (Fu et al., 2014; Fukuchi et al., 2004; Pommier et al., 2010). In CRC LXR activation suppresses colon cancer progression through inactivation of Wnt-signaling through downregulation of β-catenin (Uno et al., 2009). In addition, LXRαβ(−/−) leads to

an increase in proliferation (Vedin et al., 2013). Several studies have shown that LXR inhibits cell proliferation (Bensinger et al., 2008; Candelaria et al., 2014; Kim et al., 2010). LXRs are involved in the innate and adaptive immune responses. For example, LXRs stimulate cell clearance by macrophages, leading to apoptosis. In addition, LXRs inhibit lymphocyte proliferation, activate interferon-γ, and increase the survival rates of mice injected with tumor cells (A-Gonzalez et al., 2009; Wang et al., 2014, 2016). Treatment with an LXR agonist leads to anti-inflammatory action by downregulating inflammatory responses both in vitro and in vivo (Joseph et al., 2004).

Estrogen Receptor and SERMs

Estrogen is a sex steroid hormone made from cholesterol and secreted by the ovaries, adrenal glands, and adipose tissue. Estrogen functions in the female reproductive system and also plays a role in the heart, bones, and brain (Lewis and Jordan, 2005). Estrogens, specifically the most abundant estrogen 17-β-estradiol, bind to the estrogen receptor. Other metabolites of estradiol including estrone and estriol bind to the estrogen receptor as well but to a lower extent compared with estradiol. Estrogen binds to members of the nuclear hormone receptor superfamily known as estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ). Upon binding of estrogen to its receptor, the estrogen receptor translocates from the cytoplasm to the nucleus where it acts as a transcription factor to bind to and recruit other co-activators or co-repressors to modulate gene expression. The estrogen receptor binds to genes that contain an estrogen response element (ERE). ERα is regulated by phosphorylation. Several estrogen-responsive genes have been identified including estrogen-responsive finger protein (Efp), cytochrome c

oxidase subunit VIIa-related polypeptide (COX7RP), and the estrogen receptor-binding fragment-associated antigen 9 (EBAG9) (Inoue et al., 1993; Watanabe et al., 1998).

Both estrogen receptors have six distinct domains (A-F). The A/B domain contains a transcriptional activation domain, which interacts with transcription co-activators. Domain C has a DNA-binding domain of a zinc-finger motif that recognizes estrogen response elements (ERE), which are response elements that estrogen receptors recognize and bind to along with other transcription factors such as Fos/Jun or SP-1 (Kushner et al., 2000). The ERE are inverted palindromic sequences (Klinge, 2001). Region D is a hinge region while region E/F contains the ligand-binding domain (LBD), a domain that binds estradiol or other molecules. ERα and ERβ are highly homologous except for the N-terminal A domain (Yaşar et al., 2017). ERα is expressed in the uterus, breast, ovary, and bone, while ERβ is expressed in the central nervous system, cardiovascular system, lung, colon, and kidney. From alternative splicing, there are three ERα isoforms while ERβ has at least four isoforms. ERα regulates the genes that control cell growth, while ERβ regulates the genes involved in cell cycle progression (inhibiting cell proliferation) and apoptosis and can alter the effects of ERα by inhibiting 70 percent of ERα-regulated genes (Jia et al., 2015).

In addition to binding to and regulating genes with EREs, estrogen receptors can also regulate genes without EREs through an additional DNA-binding transcription factor that navigates ER to the gene, also known as ER-indirect DNA association genes (O'Lone et al., 2004). The main facilitator of this action is stimulating protein 1 (Sp-1). Some genes that are regulated by Sp-1 and ER are low-density lipoprotein (LDL) receptor, endothelial nitric oxide synthase (eNOS), c-fos, and cyclin D1. Other types of

DNA mediators include the c-rel subunit of NF- κB, the cAMP response element-binding protein (CREB), and nuclear transcription factor Y (O'Lone et al., 2004).

In addition to playing a role in the transcriptional activation/repression of several genes, the estrogen receptor also has rapid non-genomic effects, referred to as cell membrane-initiated signaling. In the cell membrane, E2 binds to ER, leading to activation of G-proteins (Aronica et al., 1994; Pietras and Szego, 1977; Razandi et al., 1999). For example, estrogen signaling can lead to phosphorylation of several proteins including the p38-MAPK-2 kinase pathway whose downstream effect leads to modification of heat shock protein 27 (Razandi et al., 2000). Estradiol can also activate other signaling pathways. For example, estradiol has been shown to induce PI3K-AKT signaling pathways to upregulate Cox-2 (Pedram et al., 2002). Estradiol can signal through ERK signaling pathways, leading to activation of c-fos and prolactin synthesis (Duan et al., 2002; Watters et al., 1997, 2000).

Estrogen receptors regulate colon cancer progression with estrogen lowering one's risk of developing CRC. ERβ is found mainly in the colon and is expressed at lower levels in colon cancer than in the normal colon, suggesting a protective role of ERβ in CRC. This was demonstrated when knockdown of ERβ led to an increase in cancerous polyps in an APCMin+ mouse model of colon cancer (Giroux et al., 2008). Additionally, ERβ downregulates IL-6 in colon cancer, leading to an anti-inflammation response (Edvardsson et al., 2011).

27-hydroxycholesterol is a selective estrogen receptor modulator or SERM, which are compounds that act as agonists or antagonists for the estrogen receptor depending on the tissue type (Figure 5). A classic example is tamoxifen, which acts as an antagonist in

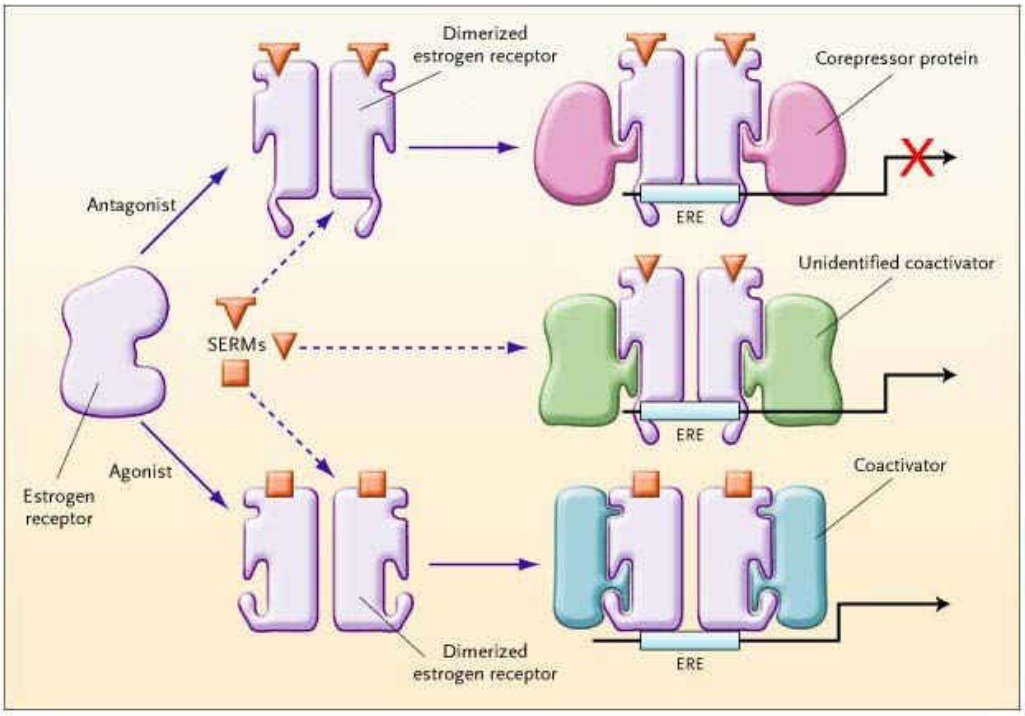

Figure 5. Selective estrogen receptor modulation in an estrogen response element-dependent and ERE-independent manner. Each class of SERMs (orange symbols) has a slightly different shape, although all will bind to the estrogen receptor. When it binds to an estrogen, antiestrogen, or SERM, the estrogen receptor undergoes a conformational change that permits its spontaneous dimerization and facilitates the subsequent interaction of the dimer with estrogen response elements (EREs) located within target genes. The estrogen-receptor-ligand complex also leads to binding of various coregulatory proteins that vary with its conformational structure. Some estrogen-receptor-SERM complexes favor corepressor recruitment (red) that, in a given target cell, increases its antagonist activity, and others favor coactivator recruitment (blue) that increases its agonist activity. Some SERMs may also facilitate the interaction of the estrogen receptor with yet-to-be-identified coactivators (green) with which estrogens or antiestrogens would not normally couple. It has now been determined that estrogen facilitates the interaction of the estrogen receptor with coactivators. The antagonist-activated estrogen receptor, on the other hand, interacts preferentially with corepressors. The binding of the different SERMs to the receptor permits the receptor to adopt conformational states that are different from each other and also distinct from that induced by classic estrogen agonists or antagonists. The implication of this model is that SERM activity will be influenced by the relative levels of expression of the coregulatory proteins (corepressors and coactivators) that are expressed in different target cells. Reproduced with permission from Riggs and Hartmann, 2003, Copyright Massachusetts Medical Society.

breast tissue and as an agonist in bone tissue. This effect leads to a decrease in estrogen receptor-positive breast cancer and a decrease in bone mineral density loss. However, tamoxifen can also act as an agonist in the uterus, leading to polyp production and increasing a women's risk of developing endometrial cancer (Archer, 2011).

27-OHC along with 24S-hydroxycholesterol and 25-hydroxycholesterol downregulate estradiol activation of ERα and β, and 27-OHC is the most powerful oxysterol with an IC50 of around 1μM. 27-OHC is able to inhibit ERβ more than ERα. Using a two-hybrid assay, it was determined that increasing 27-OHC disrupts the E2-dependent interaction between ERβ and SRC-1 but not ERα. 27-OHC inhibits the E2-dependent upregulation of Nos2 both in vitro and in vivo (Umetani et al., 2007). 27-OHC also inhibits the nontranscriptional activation of eNOS in bovine aortic endothelial cells (BAECs). Additionally, 27-OHC inhibits reendothelialization or making new endothelium cells (Umetani et al., 2007).

27-OHC is a SERM that functions as an estrogen receptor agonist in HepG2 and HeLa cells (DuSell et al., 2008). 27-OHC allows for ERα to be recruited to DNA response elements and induces ERα-dependent proliferation as a partial agonist by upregulating cyclin D1. Peptide-binding experiments showed that 27-OHC elicits a binding motif similar to those between ERα and E2 as well as ERα and tamoxifen. 27-OHC has been shown to promote MCF-7 xenograft growth in mice (Wu et al., 2013) and leads to ER-dependent cell proliferation. 27-OHC also increases ER-dependent growth (Nelson et al., 2013).

AKT

AKT, also known as protein kinase B (PKB), is a serine-threonine kinase that is involved in cell cycle progression, protein synthesis, and cellular survival. AKT contains an N-terminal plectstrin homology (PH) domain, a kinase domain, and a C-terminus regulatory domain (Song et al., 2005). The three isoforms of AKT are AKT1 (PKBα), AKT2 (PKBβ), and AKT3 (PKBγ), and they share eighty percent protein sequence identity. AKT is mostly found in the heart and lungs, whereas AKT2 is found in skeletal muscles and AKT3 is found in kidney and brain (Brodbeck et al., 1999).

AKT becomes fully activated upon phosphorylation of Threonine 308 at the catalytic kinase and Serine 473 at the C-terminus. Phosphorylation is controlled through phosphatidylinositol 3 (PI3) kinase (Alessi et al., 1996). Specifically, PI3K localizes to the plasma membrane where it converts PIP2 (phosphatidylinositol 4,5-bisphosphate) to PIP3 (phosphatidylinositol 3,4,5 trisphosphate). PIP3 recruits AKT and PDK1 kinase to the plasma membrane where PDK1 phosphorylates Threonine 308, while Serine 473 can be phosphorylated by mTORC2 (mammalian target of rapamycin complex 2) (Sarbassov et al., 2005), integrin-linked kinase (ILK) (Delcommenne et al., 1998), and mitogen-activated protein kinase-activated protein kinase-2 (MAPKAPK2) (Balendran et al., 1999). AKT can be activated through PKA (Filippa et al., 1999), calcium/calmodulin (Yano et al., 1998), and other molecules such as Hsp90 and Geb10 (Song et al., 2005). AKT is inactivated through downregulation of the PI3K pathway by PTEN via dephosphorylation of PIP3 (Maehama and Dixon, 1998). Another class of proteins that leads to dephosphorylation of AKT at serine 473 is PHLPP (PH domain and leucine-rich repeat protein phosphatase).

After being activated through phosphorylation, AKT is then free to activate a variety of molecules involved in cell cycle progression, genomic instability, and glucose metabolism. One AKT target is c-myc, which is phosphorylated by GSK-3β, causing cell cycle progression through upregulation of cyclins and downregulation of p21, p27, and p15 (Gartel and Shchors, 2003). Additionally, AKT causes phosphorylation of Rb, leading to activation of E2F. Another AKT target involved in cell cycle progression is glycogen synthase 3β, which is phosphorylated by AKT and subsequently inhibited. AKT phosphorylates and inactivates p21 at Threonine 145 and Serine 146 and downregulates p27 (Medema et al., 2000; Zhou et al., 2001). Another target of AKT is serine 166 and 186 of Mdm2, which allows for p53 ubiquitination and degradation (Ogawara et al., 2002). This allows for G1/S transition through the cell cycle. AKT phosphorylates Cdc25B (Baldin et al., 2003), Wee1A (Katayama et al., 2005), Myt1 (Okumura et al., 2002), and cdk2 (Maddika et al., 2008) to undergo G2/M transition.

AKT can also be activated by DNA damage through ATM, ATR, and DNA-PK. While ATM, ATR (Caporali et al., 2008), and DNA-PK (Bozulic et al., 2008) bind to AKT, there is no evidence that these proteins phosphorylate AKT directly. Hence, there may be other intermediate proteins involved in direct phosphorylation. Exposure to genotoxic stress or DNA damage leads to activation of Chk1 80 and inactivation of Chk2 103, which halts G2 arrest. AKT is also involved in nonhomologous end joining (NHEJ) and homologous recombination repair (HRR). Downstream targets of AKT include phosphorylation of Chk1 (Xu et al., 2010), DNA topoisomerase 2-binding protein 1 (TopBP1) (Liu et al., 2006), Brac1 (Plo et al., 2008), and Ribosomal protein S3 (RPS3) (Hegde et al., 2007), leading to DNA repair, recombination, and checkpoint signaling.

Another target of AKT is forkhead Box O family transcription factors (FoxO), which is involved in regulating apoptosis, cell cycle arrest, and catabolism through the FAS ligand gene. AKT phosphorylation leads to suppression of FoxO (van der Vos and Coffer, 2011). Another AKT target is Tuberous Sclerosis Complex 2 (TSC2), which regulates mTORC1. The function of TSC2 is to promote cell growth. AKT phosphorylates and inhibits TSC2, which activates mTORC1 (Saxton and Sabatini, 2017). AKT can also directly phosphorylate and activate mTORC1 at Serine 2448 (Navé et al., 1999). In addition, proline-rich AKT substrate of 40 kDa (PRAS40) is also phosphorylated by AKT, leading to downregulation of mTORC1 (Sancak et al., 2007). AKT also activates mTOR kinase, which leads to the phosphorylation of p70 S6 kinase (p70 S6K) and eIF4E binding proteins, resulting in cap-dependent translation (Ruggero and Pandolfi, 2003). Additionally, AKT phosphorylates and inactivates BAD and procaspase-9 (proapoptotic factors). It also prevents the release of cytochrome c (Carnero and Paramio, 2014) (Figure 6).

AKT cross-talks with other signaling molecules such as those involved in the RAS-ERK and AMPK pathways (Manning and Toker, 2017). In cancer, there is hyperactivation of AKT through amplification of EGFR, HER2, and PDK1, along with a loss of tumor suppressor genes such as PTEN and PHLPP. AKT itself can be mutated as well, including at E17K in the PH domain (Carpten et al., 2007). AKT mutations are also found in other diseases such as autoimmune diseases and neurological diseases.

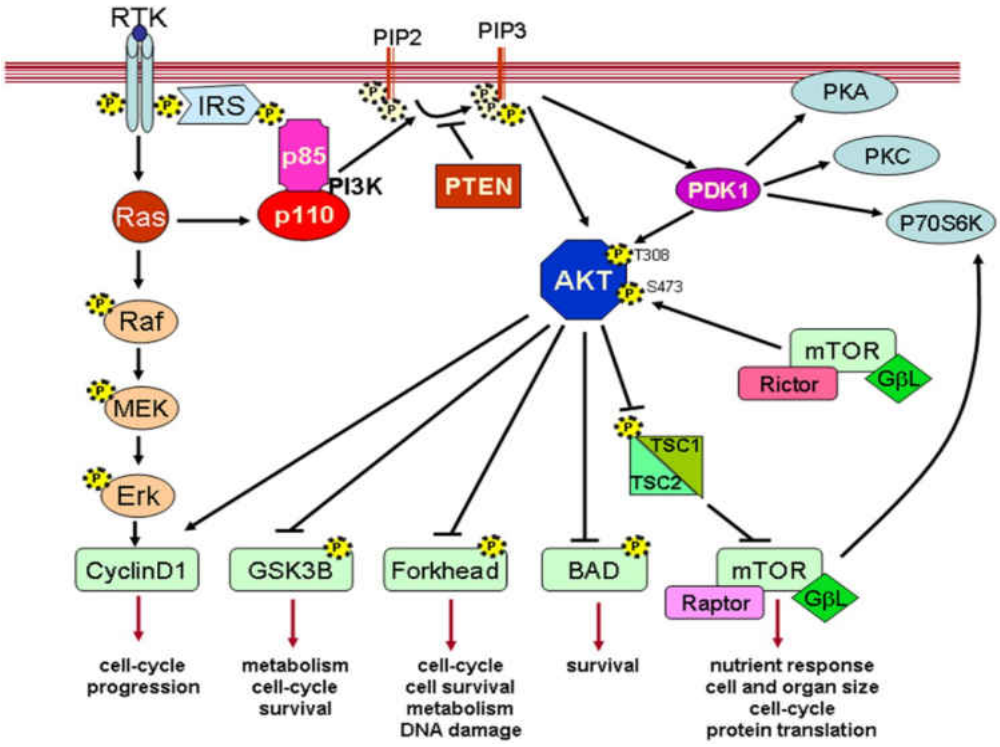

Figure 6. AKT signaling pathway. A schematic of prominent AKT signaling pathways. Reproduced with permission from Carnero and Paramio, 2014.

Insulin-Like Growth Factor (IGF)

Insulin-like growth factors are members of a family of insulin-related peptides that include IGF-1 and IGF-2. They are small peptides consisting of only 70 amino acids. IGF-1 has two chains, A and B, which are connected by a disulfide bond. IGFs bind to their receptors IGF1R and IGFR2, which are tyrosine kinase cell surface receptors. IGFs are bound by IGF-binding proteins (IGFBPs) with 90 percent of IGF1 bound to IGF-binding protein 3 (IGFBP3). IGF1 and 2 share 62 percent homology. IGF-1 is a protein that is secreted by many tissues with most action occurring in the liver. IGF-1 levels increase during fetal development and puberty but decline during adulthood, while IGF2 mainly functions during fetal development (Daughaday and Rotwein, 1989). IGFs and

their receptors signal through Ras/Raf/MAP kinase and the phosphoinositide 3 kinase/ AKT pathway. IGFs stimulate growth, proliferation, and differentiation and downregulate apoptosis (Fürstenberger and Senn, 2002).

IGF-1 promotes cellular transformation resulting from antiapoptotic activity. This is a result of p53 (see above) inducing IGFBP3. Additionally, IGFR1 plays a role in invasion, metastasis, and angiogenesis via IGF-1 (Shigematsu et al., 1999) through upregulation of hypoxia inducible factor 1α (HIF1α), which then upregulates vascular endothelial growth factor (VEGF) (Zelzer, 1998). There is a link between serum concentrations of IGF-1 and an elevated risk of breast (Maor et al., 2007), prostate (Shi et al., 2001), and colorectal cancers (Bruchim et al., 2009). Cancer treatments involve lowering the serum levels of IGFs through IGFR1 blocking antibodies, small molecule IGF1R kinase inhibitors, and siRNA against IGFR1 (Bruchim et al., 2009).

Several studies suggest a link between IGFs and CRC. Normal colon tissue has low expression levels of IGF1 and IGF2 mRNA, while the expression levels in colon cancer tumors are 3- to 50-fold higher (Tricoli et al., 1986). A study of Caco2 colon cancer cells found that IGF2 RNA levels and IGFR1 levels were higher when cells were proliferating rather than dormant (Zarrilli et al., 1994). Additionally, patients with acromegaly exhibit an approximate 10-fold increase in IGF1 levels and have a higher risk of developing colon and rectal cancers (Baris et al., 2002). A meta regression study found a positive correlation between IGF1 and CRC risk (Renehan et al., 2004).

CHAPTER II

27-OHC DECREASES CELLULAR PROLIFERATION INDEPENDENTLY OF LXR AND ER

Introduction and Rational

A major aspect of cancer cells is rampant cellular proliferation. Cell proliferation occurs when cells begin to double in number through cell growth and cell division, where DNA is replicated and sister chromatids are separated to create two daughter cells. Cell division consists of several stages including the G1 phase, S phase, G2 phase, and M phase. The G1 phase is when cellular growth occurs, while the S phase is where DNA is synthesized. The G2 phase allows for proofreading and packaging of the DNA in order to prepare for the M phase or mitosis in which chromosomes segregate and cytokinesis occurs. Mitosis is divided into prophase, metaphase, anaphase, and telophase or cytokinesis. The cell cycle is regulated positively through cyclin-dependent kinases (Cdks), which phosphorylate a variety of substrates and contain a kinase subunit and a cyclin substrate. For example, in order to go through G1 and then into S phase, cyclin E needs to be transcribed, which requires the transcription factor E2F. During early G1 phase, E2F is bound to and inhibited by Rb, which is inhibited through phosphorylation. As the cells go through G1, there is an accumulation of G1-cdk (Cdk4-cyclin D), which leads to phosphorylation of Rb, which removes E2F and allows for cyclin E to be produced and bind to Cdk2. To enter into G2 phase, there needs to be an accumulation of cyclin A that binds to Cdk2, while progression into M phase requires cyclin B binding to

Cdk1. Several Cdk inhibitors also exist including p16, p21, and p27. They inhibit progression through the cell cycle by binding to Cdk-cyclin subunits and obstructing the kinase subunit, therefore preventing its activity (2007) (Figure 7).

The cell cycle can also be regulated by cellular signaling through mitogens and anti-mitogens. One example of a mitogen is EGF or epidermal growth factor. EGF binds to the EGF receptor on the extracellular membrane, and the dimers on the receptors phosphorylate and activate each other. This functions as a binding site for proteins that contain SH2 domains, such as Grb2. Grb2 recruits a guanine nucleotide exchange factor

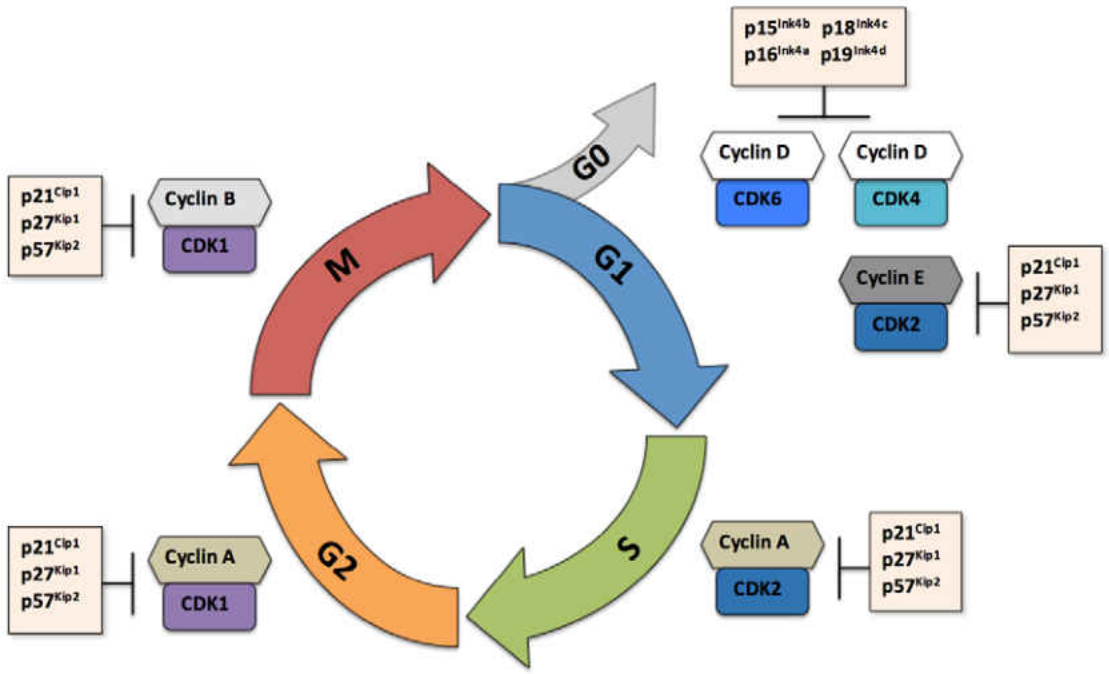

Figure 7. Cyclins and CDKs regulate the cell cycle. In eukaryotic cells, there are multiple CDK-cyclin complexes that play specific roles at various phases in the cell cycle. These complexes include three interphase CDKs (CDK2, CDK4, and CDK6), a mitotic CDK1, and ten cyclins belonging to four different classes (A-, B-, D-, and E-type cyclins), where mitogenic signals correlate with the increasing expression of D-type cyclins. Reproduced with permission from Lutful Kabir et al., 2015.

(GEF) known as Sos. Sos activates RAS through the exchange of GDP to GTP. RAS then activates a signal transduction pathway through activation and phosphorylation of Raf, which activates and phosphorylates MEK and then phosphorylates and activates MAP. MAP then activates a variety of transcription factors that help cells proliferate, such as myc. Other mitogenic pathways include Wnt signaling (see above) (2007).

Several studies have implicated oxysterols in several different types of cancers including breast and prostate cancers (Hashimoto et al., 2016; Raza et al., 2016, 2017). Oxysterols have also been involved in several diseases, such as atherosclerosis (Umetani et al., 2014), Alzheimer's disease (Lütjohann et al., 2000; Mateos et al., 2011), and Huntington's disease (Leoni et al., 2013).Oxysterols play a role in cancer cell proliferation both through proproliferative and antiproliferative effects depending on the oxysterol and the type of cancer. Similar to our findings with 27-OHC, the oxysterol 7-beta hydroxycholesterol has been shown to decrease cell proliferation in Caco2 cells (Roussi et al., 2005). Additionally, treatment of Caco2 cells with another oxysterol, 25-hydroxycholesterol, led to increased IL-8 and IL-1β levels (Bai et al., 2005). However, no studies to date have investigated the role of 27-OHC in cell proliferation in colorectal cell lines, as this oxysterol is found in the plasma. We therefore investigated the role of 27-OHC in CRC using two colon cancer cell lines: Caco2 and metastatic SW620.

Material and Methods

Materials

Caco2 cells (cat # HTB-37™), MTT Cell Proliferation Assay (cat # 30-1010K), and SW620 cells (cat # CCL-227™) (ATCC, Manassas, VA). DMEM media, Leibovitz's L-15 media, 100U/ml penicillin, 100ug/ml streptomycin, and 0.25ug/ml amphotericin

(Life Technologies, Carlsbad, CA). FBS (Atlanta Biologicals, Flowery Branch, GA). GW3965 (cat # 2474), 27-hydroxycholesterol (cat # 3907), and estradiol (cat # 2824) (R&D systems from Minneapolis, MN). 6α-epoxycholesterol-3-sulfate (cat # C4136-000) (Steraloids, Newport, RI). CytoTox 96® non-radioactive cytotoxicity assay (cat # G1782) and DeadEnd Florometric TUNEL assay (cat # G3250) (Promega, Valencia, CA). Hard Set mounting medium with DAPI (cat # H-1500) (Vector Laboratories, Burlingame, CA). DNase (cat #AM2222), Alexa Fluor 594nm (cat #A11037), Halt™ proteases and phosphatase inhibitor cocktail (cat #78446) (Fisher Scientific, Hampton, NH). Immun-Blot® PVDF membrane for protein blotting (cat # 1620177), Clarity™ Western ECL substrate (BioRAD, Hercules, CA). The antibodies used in this study are included in Table 1. Our study was approved by the Institutional Biosafety Committee of the School of Medicine at the University of North Dakota.

Cell Culture

Caco2 non-metastatic cells were grown in DMEM media containing 10% FBS. SW620 metastatic cells were grown in Leibovitz's L-15 containing 10% FBS; 100U/ml penicillin, 100ug/ml streptomycin, and 0.25ug/ml amphotericin were added. The cells were maintained at 5% CO_2 at 37°C. Cells were treated with 0, 0.5, 1, 10, 50, or 100 µM of 27-hydroxycholesterol (27-OHC), 2nM estradiol (E2), 100nM ICI 182 780, 10µM GW3965, and/or 10µM of 5α-6α-epoxycholesterol-3-sulfate (ECHS). Known circulating 27-OHC levels have been reported to be between 0.15–0.73 µM, and these concentrations can be in the millimolar range in some pathological situations such as atherosclerosis (Brown and Jessup, 1999).

Table 1. Antibodies used in the present study with their sources and applications.

Name	Catalog #	Species	Application
ABCG1	Fisher: PA5-13462	Rb	Western Blotting
ABCA1	Novus biologicals: 105SS	Rb	Western Blotting
ERα	Santa Cruz: sc-787	Ms	Western Blotting
ERβ	Fisher: PAI310B	Rb	Western Blotting
LXRα	Fisher: PAI330	Rb	Western Blotting
LXRβ	Fisher: PAI333	Rb	Western Blotting
AKT	Cell Signaling 9272S	Rb	Western Blotting
p-AKT	Cell signaling 4060S	Rb	Western Blotting
ERα	Fisher: PA5-16440	Rb	Immunofluorescence
IGF-1	Abcam ab36532	Ms	Western Blotting

MTT Assay

A total of 14,000 cells/well were seeded in a 96-well plate and grown overnight in Caco2 and SW620 cells. The next morning, different concentrations of 27-OHC (0-300 μM) were added, and the cells were incubated for 24 hrs. After 24hrs, an MTT assay was performed following the manufacturer's protocol. Briefly, 10ul of MTT reagent was added, and the cells were incubated at 37°C until a purple precipitate formed (2-4hrs). The media was removed, 100ul of detergent reagent was added, and the plate was incubated at room temperature in the dark for 2 hours. The plate was then read using a microplate reader at 570nm. Ethanol (ETOH) was used as a vehicle control with

treatment made to 1. The other samples were normalized to the ETOH treatment, and the fold change was determined.

LDH Assay

A total of 14,000 cells/well were seeded in a 96-well plate and grown overnight in Caco2 and SW620 cells. The next morning, different concentrations of 27-OHC (0-300 μM) were added and incubated for 24hrs. After 24hrs, the LDH assay was performed using a cytoTox 96® non-radioactive cytotoxicity assay following the manufacturer's protocol. Aliquots of media (50ul) were transferred to a new 96-well plate, and 50ul of CytoTox 96® Reagent was added for 30 minutes in the dark at room temperature. Stop solution (50ul) was then added, and the absorbance was read at 490nm. Ethanol treatment was made to 1. The other samples were normalized to the ethanol treatment, and the fold change was determined.

TUNEL Assay

To measure apoptosis, a TUNEL assay was performed using a DeadEnd Florometric TUNEL assay following manufacturer's protocol on Caco2 and SW620 cells. The cells were fixed with 4% formaldehyde on a cover slip and permeabilized with 0.2% trition X-100 solution. The cells were then incubated with the terminal deoxynucleotidyl transferase fluorescein-12-dUTP for 1 hour at 37°C in the dark. The slides were mounted using a solution containing DAPI to stain the nuclei. The slides were then visualized with a fluorescence microscopy. DNase was used as a positive TUNEL control.

Immunofluorescence

Caco2 and SW620 cells were used. A total of six slides per cell line of 100,000 cells were seeded on a coverslip, rinsed with PBS, fixed, and permeabilized with 4% formaldehyde, 0.1% trition X-100 in PBS. The coverslips were blocked with 10% goat serum. The primary antibody [anti-estrogen receptor alpha (see Table 1) at a 1:100 dilution] was added overnight at 4°C. The estrogen receptor alpha antibody was conjugated with Alexa Fluor 594nm and mounted with Hard Set mounting medium with DAPI. Slides were visualized using a Zeiss LSM-510 Meta Confocal Microscope.

Western Blotting

Caco2 and SW620 cells were harvested by a PBS wash and the addition of RIPA buffer with Halt™ proteases and a phosphatase inhibitor cocktail followed by spinning at 14,000g for 10 minutes at 4°C. The supernatant was then used for further processing. A Bradford assay using Bovine Serum Albumin (BSA) assay was used to quantify the protein concentrations of the lysates using a standard protocol. Between 10ug to 35ug of sample were separated using 10 percent (15 percent for IGF-1) SDS-PAGE gels and transferred to a PVDF membrane. The membrane was then incubated with the antibodies (see table 1). The membrane was then exposed to Clarity™ Western ECL substrate. The blots were visualized using the Omega Lum™ G imaging system (Aplegen), and the bands were quantified through densitometry using image j software.

CyQUANT Cell Proliferation Assay

To measure cell proliferation, a CyQUANT® NF Cell Proliferation assay was used following the manufacturer's protocol. Briefly, 100,000 cells were plated in a 96-well plate and treated with different concentrations of 27-OHC. After 24 hours of treatment

with 27-OHC, the media was removed and CyQUANT® was added to the wells for 1 hour. The plate was then read using a microplate reader at 485nm excitation and 530nm emission.

Statistical Analysis

All of the assays were performed in triplicates. The amount of significance was determined using either an unpaired t-test or one way analysis of variance. Statistical testing was performed using Graphpad Prism software 7. All data is presented as mean ± SEM. The values' units are relative to control.

Results

27-OHC Decreases Cell Proliferation via an MTT Assay

Two colon cancer cell lines were utilized, non-metastatic Caco2 and metastatic SW620. To determine the role of 27-OHC in CRC, cell proliferation was measured after adding different concentrations of 27-OHC for 24 hours using an MTT assay that measures cell viability. We found that with concentrations at and above 10μM, there was a significant decrease in cell proliferation relative to vehicle control (Figure 8A and 8B). Using a different cell proliferation assay, CyQuant®, we observed a similar decrease in cell proliferation with an increase in the concentration of 27-OHC (Figure 8C and 8D).

The 27-OHC-Induced Decrease in Proliferation is Independent of Cellular Cytotoxicity and Apoptotic Cellular Death

To determine whether this decrease in cell proliferation is associated with cellular cytotoxicity, an LDH assay was performed in both cell lines. We found that the 27-OHC-induced decrease in cell proliferation and cell viability was not associated with cell death or cytotoxicity at physiological concentrations in both the Caco2 cells and SW620 cells. We subsequently determined whether it was possible to elicit cytotoxicity in these cells

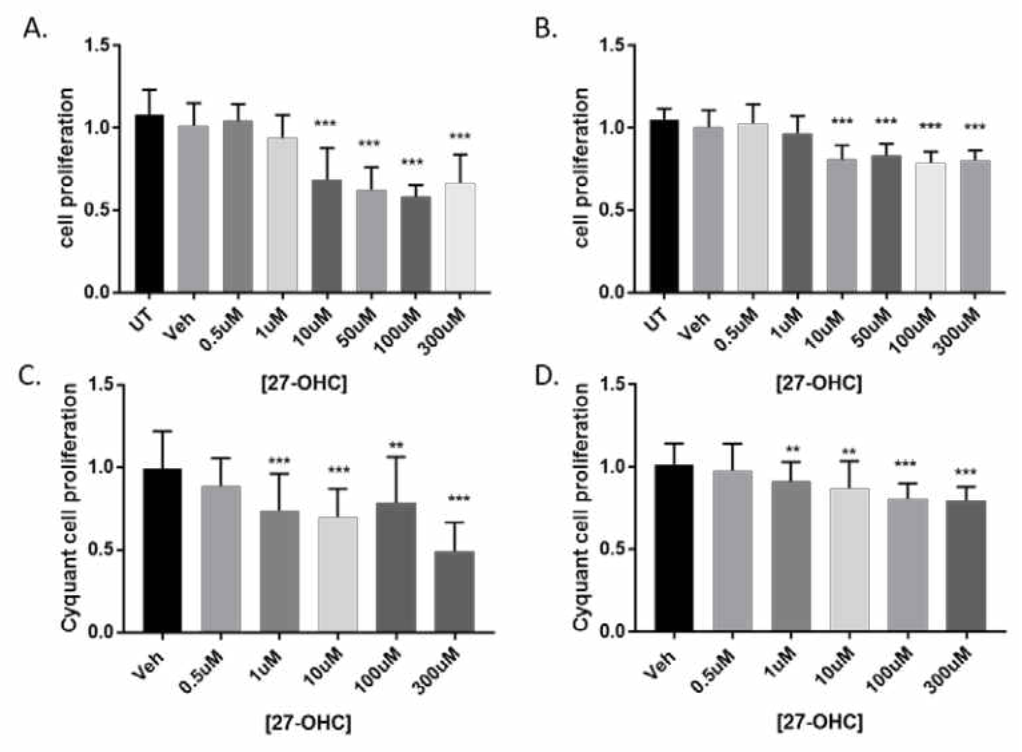

Figure 8. 27-hydroxycholesterol decreases cell proliferation in Caco2 and SW620 cells. MTT assay (A and B) and cyQuant (C and D) show that treatment with 27-OHC reduces cell proliferation in Caco2 (A and C) and SW620 cells (B and D) after 24 hours. ** $p<0.01$ and ***$p<0.001$.

with 27-OHC. We found that 27-OHC at a higher supra-physiological concentration of 300μM did cause cell death in Caco2 cells, but not SW620 cells (Figure 9A and 9B). Our data suggests that the 27-OHC-induced decrease is the result of cell proliferation and cell viability rather than cellular cytotoxicity.

To determine whether the reduction in cell proliferation emanates from significant cell death following treatment with 27-OHC, a Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed. This assay detects DNA fragmentation, which represents a characteristic hallmark of apoptosis. Our data shows that at concentrations that induced cell proliferation, 27-OHC does not induce apoptotic

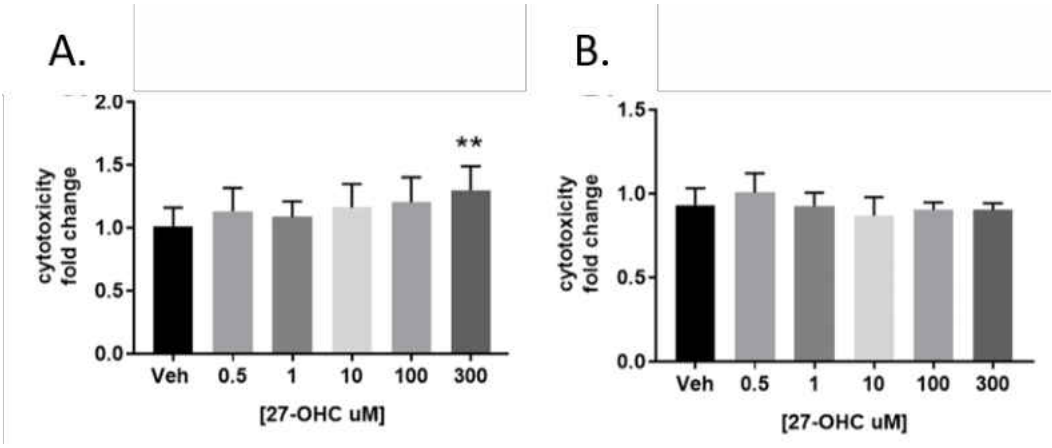

Figure 9. 27-OHC does not result in cellular cytotoxicity. The LDH assay shows that 24 hours of treatment with 27-OHC doesn't trigger cellular cytotoxicity in Caco2 (A) and SW620 (B) cells. ** p-value <0.01.

cell death in either Caco2 (Figure 10A) or SW620 (Figure 10B) cells, suggesting that the 27-OHC-induced reduction in cell proliferation is not due to apoptotic cell death.

27-OHC Decreases Cell Proliferation Independently of ER or LXR

We determined the protein levels of the 27-OHC receptors ERαβ and LXRαβ in 27-OHC-treated Caco2 and SW620 cell lines. Our data shows that both cell lines express ER and LXR, both isoform α and β. Protein levels of LXRα, LXRβ, ERα, and ERβ didn't differ between vehicle and 27-OHC treatments (Figure 11A-D).

To determine the extent to which the effects of 27-OHC on cell proliferation and cell viability emanate from the effects on LXR or ER, we treated cells with agonists and antagonists of ER and LXR. We found that while 27-OHC reduces cell proliferation and cell viability, neither the ER agonist estradiol (E2) nor the ER antagonist ICI 182 780 affects cell proliferation and cell viability. Neither the LXR agonist GW3965 nor the LXR antagonist ECHS and binds to all isoforms of the receptor affected cell proliferation and cell viability in Caco2 cells (Figure 12A) or in SW620 cells (Figure 12B) as well.

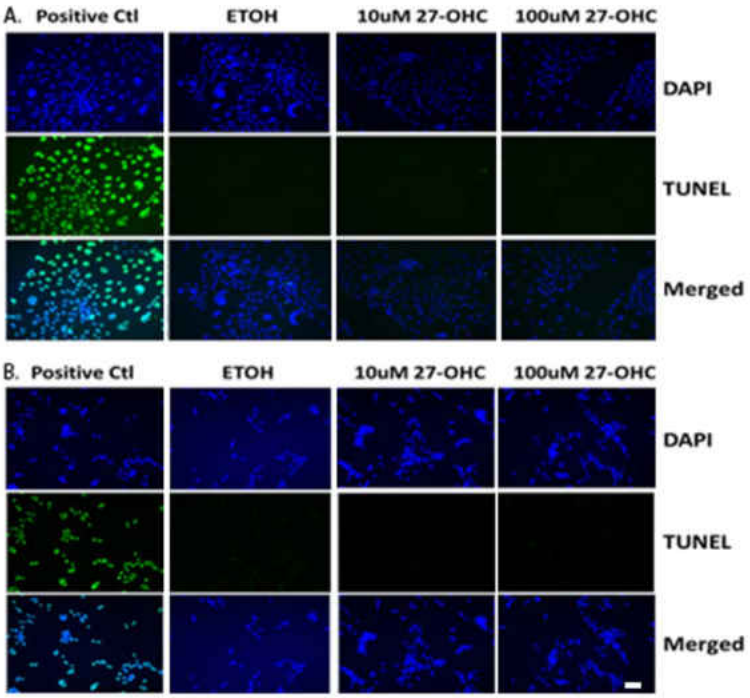

Figure 10. 27-OHC treatment doesn't cause apoptotic cell death. The TUNEL assay shows that treatment for 24h with 27-OHC doesn't trigger cell death in Caco2 cells (A) or SW620 cells (B). DAPI (blue) is a counter stain for nuclei, and DNase (green) was used as positive control.

Since changes in cellular viability and cellular proliferation are associated with changes in cytotoxicity and given that we did not observe changes in cellular viability and cellular proliferation with the agonists and antagonists of ER and LXR [as determined by MTT reduction assay (Figure 12A, B)], we did not analyze cell death cytotoxicity exhibited by these agonists and antagonists of ER and LXR. Furthermore, as the LXR agonist (GW3965) and the LXR antagonist (ECHS) did not cause any changes in cell proliferation and cell viability (Figure 12A, B), it was necessary to establish that the LXR transcriptional machinery was responsive in Caco-2 as well as SW620 cells and that the lack of effects of 27-OHC on LXR transcriptional activity were indeed valid and not a consequence of unresponsive LXR transcriptional machinery. To this end,

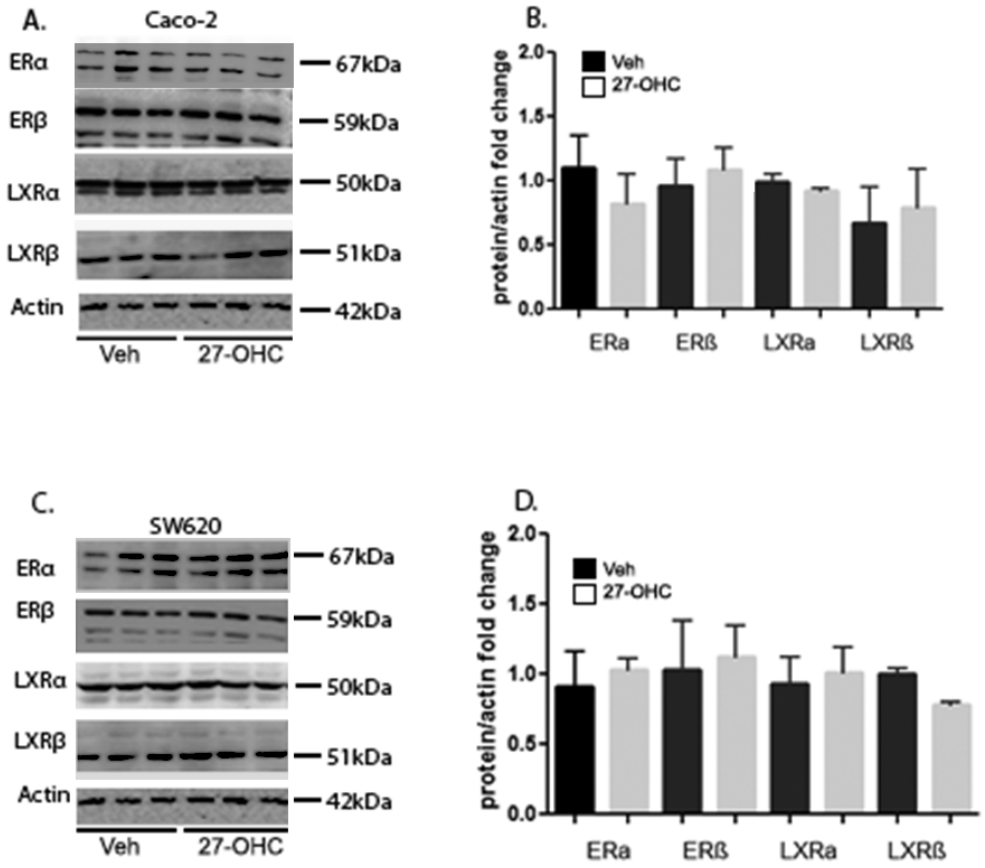

Figure 11. Both Caco2 and SW620 cells express ER and LXR. Western blot analysis shows that both Caco2 (A) and SW620 (C) cells express ERα, ERβ, LXRα, and LXRβ isoforms. Optical density analysis shows that the protein levels of ERα, ERβ, LXRα, and LXRβ are similar in both vehicle and 27-OHC (10μM) -treated Caco2 (B) and SW620 (D) cells. All blots were not significant.

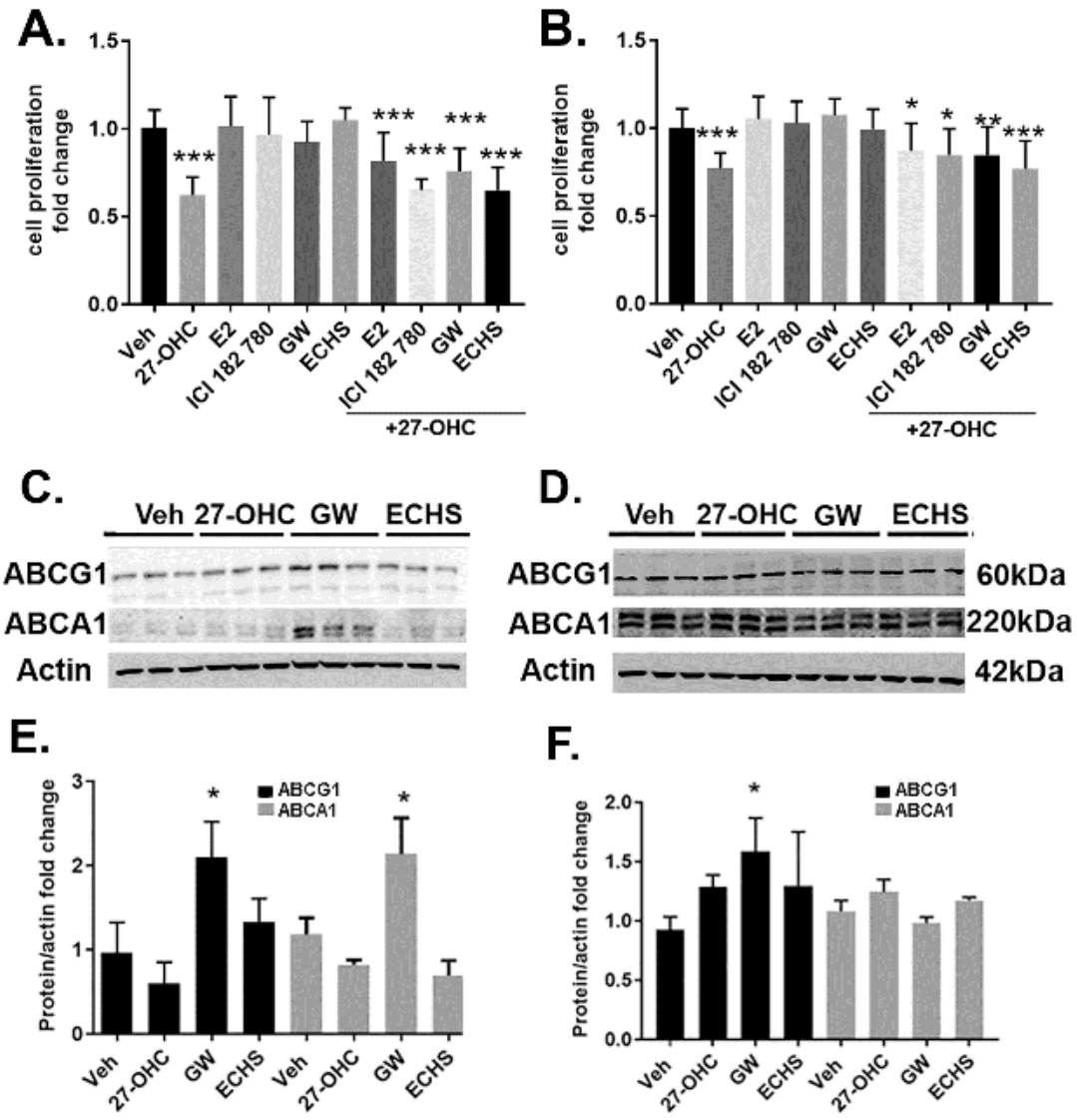

Figure 12. Treatment of Caco2 (A) or SW620 (B) cells with the ER agonist E2, the ER antagonist ICI 182 780 27-OHC, the LXR agonist GW3965, or the LXR antagonist ECHS didn't affect cell proliferation either when administered alone or in presence of 27-OHC (10 μM). Western blot and optical density analysis, respectively, show that treatment of Caco2 (C, E) or SW620 (D,F) cells with the LXR agonist GW3965 increased the protein levels of ABCG1, a downstream target protein of LXR activation, in Caco2 but not SW620 cells. *p <0.05, ** p<0.01, and *** p<0.001

we examined the effects of the LXR agonist GW3965 on the expression of the LXR target genes ABCA1 and ABCG1 (Figure 12C-F). Our data shows that the concentration of GW3965 used (10μM) was above the minimal effective concentration to induce the LXR target genes ABCA1 and ABCG1, as determined by immunoblotting and densitometric analysis (Figure 12C-F). While the LXR agonist GW3965 upregulated the protein levels of both ABCA1 and ABCG1 in Caco2 cells and only ABCG1 in SW620 cells (Figure 12C-F), the LXR antagonist ECHS did not downregulate the protein levels of ABCA1 or ABCG1 in Caco2 and SW620 cells (Figure 12 C-F). Interestingly, GW3965 at the same aforementioned concentration (10μM) did not affect cell viability and cell proliferation, as determined by the MTT reduction assay in Caco2 and SW620 cells (Figure 12A, B). This data suggests that LXR was transcriptionally responsive and that the concentration of GW3965 (10μM) was above the minimal effective concentration to activate LXR, while not inducing any changes in cellular proliferation and cell viability. To further verify whether the 27-OHC-induced effects on cell proliferation were associated with LXR activation, we examined the expression levels of the LXR target genes ABCA1 and ABCG1 in 27-OHC-treated cells. We found that 27-OHC did not upregulate the expression levels of the LXR target genes ABCA1 and ABCG1 in Caco2 and SW620 cells (Figure 12C-4F) while eliciting cell proliferation and cell viability changes (Figure 12 A,B). Our data suggests that the 27-OHC-induced decrease in cell proliferation and cell viability is independent of LXR activation.

Analogously, as the ER agonist estradiol (E2) and the ER antagonist ICI 182780 did not cause any changes in cell proliferation and cell viability (Figure 12 A, B), it was necessary to establish that the ER transcriptional machinery was responsive in Caco-2

and SW620 cells. We assessed the integrity of the ER transcriptional activity in the Caco2 and SW620 cell lines using estradiol (E2) as the positive control for ERα activation. We determined the estradiol-induced nuclear localization of ERα as a degree of ER activation as well as a measure for demonstrating that ER signaling is responsive in these cell lines. We found that estradiol (2nM) elicited a pronounced translocation of ERα from the cytosol into the nucleus in Caco2 cells and SW620 cells (Figure 13A, arrows), demonstrating that the ER signaling was responsive in these cell-lines as well as that the concentration of estradiol was above the minimal effective concentration. However, 27-OHC did not elicit any effect on the translocation of ERα from the cytosol into the nucleus (Figure 13A, B), thereby demonstrating that 27-OHC elicited no effects on the activation status of the ER. Our data suggests that the 27-OHC-induced decrease in cell proliferation and cell viability is independent of ER activation.

27-OHC Affects AKT Signaling in Caco2 Cells but not SW620 Cells

Because AKT is a signaling molecule involved in regulating cellular proliferation, we determined the extent to which AKT signaling is involved in the 27-OHC-induced decrease in cell proliferation. We assessed the protein levels of the activated, phosphorylated form of AKT (p-AKT at serine 473), along with AKT levels. Our data shows a significant decrease in p-AKT and increase in total AKT levels in Caco2 cells treated with 27-OHC compared with vehicle treatment (Figure 14 A-B). In SW620 cells, there was no significant difference in p-AKT or AKT levels (Figure 14 A-B). To determine the functional consequence of the 27-OHC-induced reduction in p-AKT levels, we determined whether AKT inhibition affects cell proliferation and cell viability. To this

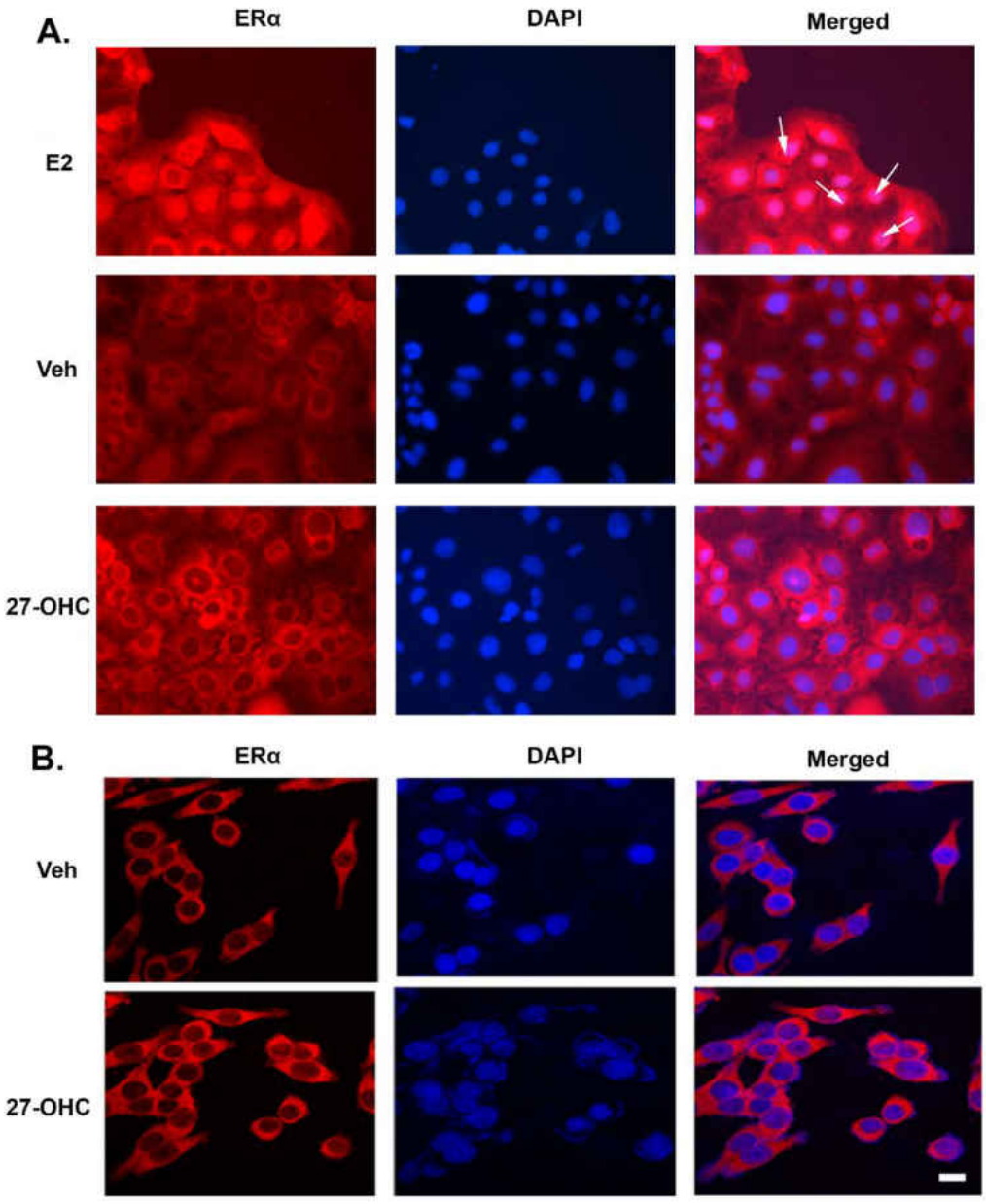

Figure 13. 27-OHC did not induce estrogen receptor nuclear translocation. Treatment of Caco2 (A) and SW620 (B) cells with 27-OHC (10μM) didn't lead to the translocation of the ER from the cytoplasm to the nucleus, as evidenced by the presence of immunoreactivity to the ER antibody (red) in the cytoplasm but not in the nucleus (counterstained with DAPI (blue).

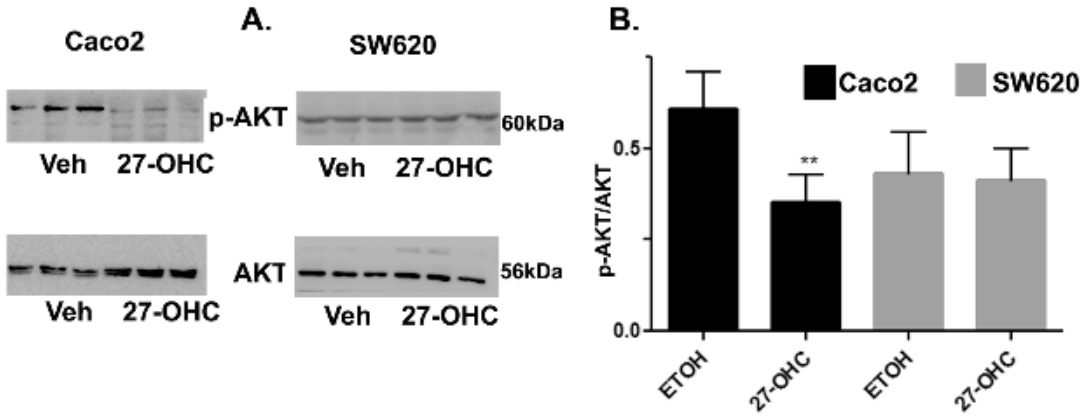

Figure 14. 27-OHC affects p-AKT expression in Caco2 cells but not SW620 cells. Western blot analysis (A) and optical density analysis (B) showing reduced levels of active p-AKT. ** p-value < 0.01.

end, we used the pan AKT inhibitor, Afuresertib, which competitively inhibits AKT kinase activity. We found that the AKT inhibitor Afuresertib significantly decreases the proliferation and cell viability of Caco2 cells, as determined by an MTT reduction assay (Figure 15A). Furthermore, we found that the AKT inhibitor Afuresertib caused a decrease in total AKT levels, as determined by immunoblotting and densitometric analysis (Figure 15B, C). Additionally there was an increase in the p-AKT at serine 473 with the addition of Afuresertib (Figure B, D, and E). Our data shows that AKT activation plays a critical role in the cell proliferation and cell viability of Caco2 cells and could be an underlying target of the 27-OHC-induced decrease in cell proliferation and cell viability.

27-OHC Doesn't Affect IGF-1 Levels

Since insulin-like growth factor is involved in proliferation and it has been shown that 27-OHC mediates IGF-1 levels, we wanted to determine the impact of 27-OHC on IGF-1 in colon cancer cell lines. To do this, we measured the amount of secreted IGF-1 in

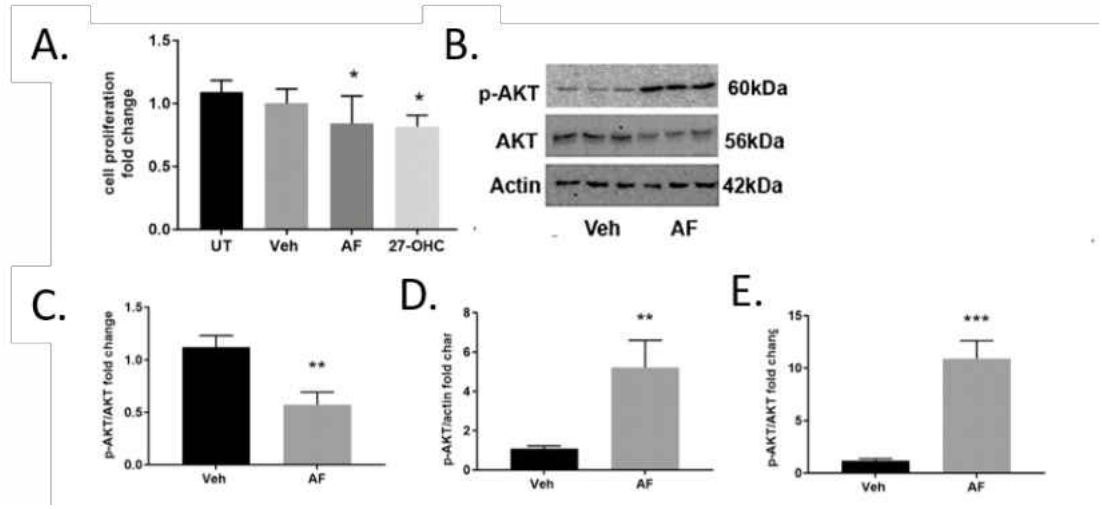

Figure 15. The AKT inhibitor Afuresertib decreases cell proliferation. (A) Cell proliferation was measured with an MTT assay with 1µM afuresertib and 10µM of 27-OHC in Caco2 cells. (B) Western blot of Caco2 cells showing that the AKT inhibitor afuresertib decreased AKT levels with the quantification in (C-E). * is p-value <0.05. ** is p-value < 0.01. *** is p-value <0.001.

the media through western blot analysis after treatment with 10µM of 27-OHC (the lowest concentration that elicited cell proliferation changes through the MTT assay) for 24 hours. We found that there was no significant difference in secreted IGF-1 levels after 27-OHC treatment in both colon cancer cell lines (Figure 16).

Discussion and Future Directions

In this study, we determined the effects of the oxysterol 27-OHC on cell proliferation in Caco2 and SW620 colorectal cancer cells. The MTT assay shows that while physiological concentrations of 27-OHC didn't affect proliferation, supra physiological concentrations (10, 50 and 100µM) substantially reduced basal cell proliferation. The reduction in cell proliferation with 27-OHC was not associated with

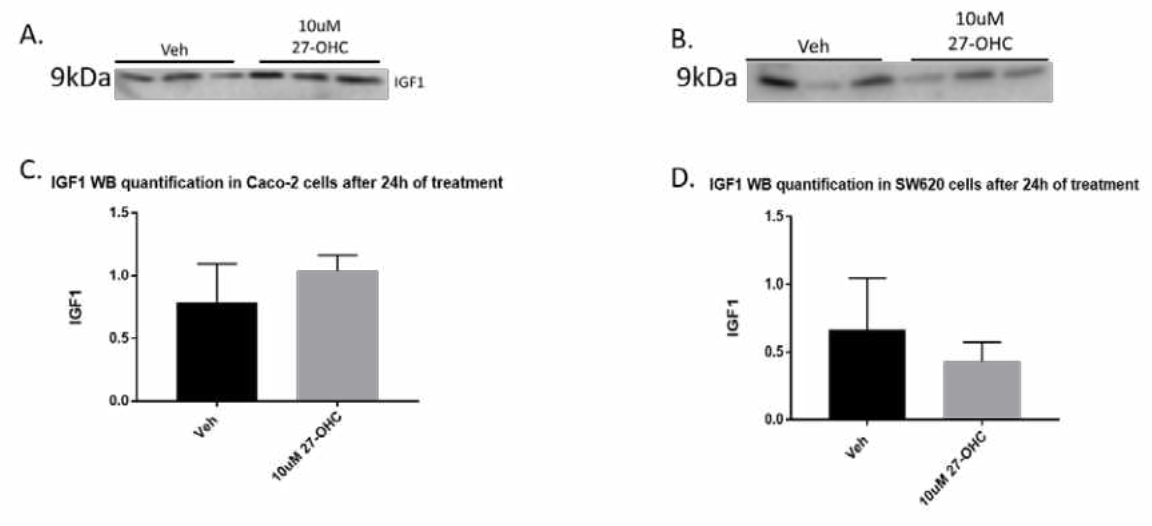

Figure 16. 27-OHC doesn't affect IGF-1 levels in Caco2 and SW620 cells. Caco2 (A) and SW620 (B) were treated with 10μM of 27-OHC. The media was collected and concentrated, and western blot for IGF1 was performed. All blots were not significant.

cytotoxicity or apoptotic cell death. The effects of 27-OHC on proliferation appear to be independent of its nuclear receptors LXR or ER. We also show that in Caco2 but not SW620 cells, the reduction in cell proliferation is associated with a decrease in p-AKT levels.

Colorectal Cancer can develop after periods of chronic inflammation, such as inflammatory bowel diseases including Crohn's disease (Jewel Samadder et al., 2017). Oxysterols, which have been shown to be involved in both innate and adaptive immunity, increase the levels of inflammatory cytokines and chemokines such as TNF-α, Il-1β, and Il-8. Oxysterol synthesis is a component of the regulatory network that modulates the extent of innate immune reactions as well as the nature and intensity of adaptive responses (Park and Scott, 2010). Inhibition of 25-hydroxylase in macrophages increases IL-1 via SREBP downregulation (Reboldi et al., 2014). 25-hydroxycholesterol has also been shown to regulate B cell proliferation and IgA levels (Bauman et al., 2009).

Furthermore, 27-hydroxycholesterol has been shown to inhibit viral infections (Civra et al., 2015).

27-OHC has been shown to act as a selective estrogen receptor modulator (SERM) (DuSell et al., 2008; Umetani and Shaul, 2011; Umetani et al., 2007). There appears to be a protective association between hormone replacement therapy and colorectal cancer, specifically through activation of ERβ (Barzi et al., 2013). For example, transfection of the colon cancer line SW489 with ERβ resulted in a decrease in cell proliferation and cell cycle arrest with xenographs weighing 70% less than controls (Hartman et al., 2009). 27-hydroxycholesterol is also a ligand for the liver-x-receptor (Fu et al., 2001), which is known to play a role in colon cancer progression. Indeed, it has been shown that LXR activation through addition of the agonist GW3965 reduces proliferation in the human colorectal cancer HT29 cell line (Lo Sasso et al., 2013). However, this effect was not seen in Caco2 or SW620 cells. Administration of the LXR specific agonist GW3965 also significantly reduces proliferation in the mouse colon (Vedin et al., 2013).

Our results also demonstrate that treatment with the ER agonist estradiol did not influence cell proliferation in both cell lines, suggesting that 27-OHC-induced cell proliferation in CRC cells is independent of effects on the ER. Previous studies showed that E1, but not E2, significantly decreases proliferation when added exogenously to the colonic epithelial cell line SW620 (English et al., 1999). In different cell models, however, 27-OHC has been shown to increase proliferation in the endometrium through the ER and LXR pathways (Gibson et al., 2018). Although we show a decrease in cell proliferation, the cellular mechanisms by which 27-OHC affects proliferation are yet to

be determined. As the effects of 27-OHC on proliferation appear to be independent of the 27-OHC receptors LXR and ER, other signaling pathways such as the AKT pathways may be involved in the effects of 27-OHC.

We show a decrease in p-AKT levels following 27-OHC treatment in Caco2 but not SW620 cells. Our current results are in accordance with previous data showing reduced p-AKT levels in organotypic hippocampal slices from mice incubated with 27-OHC (Sharma et al., 2008). Other labs have also shown that 27-OHC leads to a decrease in p-AKT levels (Ismail et al., 2017). In other cell models, however, such as macrophages and lymphocytes, 27-OHC has been shown to increase p-AKT levels (Kim et al., 2014; Vurusaner et al., 2014). Whether the effects on p-AKT mediate the effect of 27-OHC on cell proliferation in Caco2 cells has yet to be confirmed, and the cellular mechanisms mediating 27-OHC-induced proliferation in SW620 cells have yet to be identified. There could be a variety of targets for the 27-OHC-mediated regulation of AKT signaling. Some upstream targets include PTEN, PI3K, PKA (Filippa et al., 1999), calcium/calmodulin (Yano et al., 1998), and other molecules such as Hsp90 and Geb10. Additionally, downstream targets include NF-κB and GS3-Kβ. It would be interesting to determine the molecular mechanism of 27-OHC in AKT signaling in these cell lines.

Several different assays exist for cell proliferation. The most sensitive method includes incorporation of 3H-thymidine into proliferating cells, which can then be measured with a scintillation counter. A drawback to this method is the use of radioactivity. A safer, alternative method involves incorporation of 5'-bromo-2'-deoxyuridine (BrdU), which can be read through a colorimeteric assay or chemiluminescent assay. Another type of method to measure proliferation is metabolic

assays, which include MTT, XTT, MTS, and WST1. When tetrazolium salts become reduced in the presence of proliferating cells, they produce formazan dye that in the presence of a detergent changes the color of the cultured media, which can be read using spectrophotometer or microplate reader. A drawback is that this is an endpoint type of experiment and a final read-out. Another method of measuring cell proliferation is to measure ATP inside the cell, as dying cells have little ATP. This creates a linear relationship between cell number and ATP amounts, and the ATP can be measured using a luciferase assay. Other assays include detection of proliferation markers including Ki-67, PCNA, and topoisomerase IIB.

Several assays for assessing cellular cytotoxicity exist. A common method involves LDH or lactate dehydrogenase, which is a soluble enzyme that is released into the extracellular space when the cell and the membrane are damaged. LDH in the media then produces NADH and H^+ through the conversion of lactate to pyruvate. The H^+ is then transferred to the tetrazolium salt to produce a red formazan salt. This is a simple and reliable method. Another method involves the measurement of Cr^{51}, which is more sensitive than an LDH assay but uses radioactivity. Other methods include the use of membrane-impermeable fluorescent dyes such as 7-AAD and DRAQ7™ or other stains with amine-reactive dyes that can bind to cells with broken membranes through interaction with intracellular amines.

Several apoptosis assays exist including the TUNEL assay or Terminal dUTP Nick End-Labeling, which measures endonuclease cleavage. A terminal transferase is used to add a labeled UTP to the 3'-end of DNA, which can be analyzed with a fluorescence microscope. This assay can lead to false positives if the cells are undergoing

DNA repair, replication, and transcription. Another technique is the DNA laddering technique that visualizes DNA fragmentation of lysed cells processed with agarose gel electrophoresis. Some disadvantages of this technique include false positives from necrotic cells, and it shouldn't be used if there are low numbers of apoptotic cells. Another technique is real-time PCR of the genes involved in apoptosis such as those coding receptors, transcription factors, and ligands. Detection of FITC-labeled annexin V, a protein that binds to phosphatidylserine, through fluorescent microscopy can also be used to detect apoptosis. It is highly sensitive but can also bind to necrotic cells, which can be distinguished by looking at membrane integrity. This also can be compensated for by using dyes such as propidium iodide and trypan blue, which do not label necrotic cells. To detect early changes in apoptosis, the release of cytochrome c can be measured through fluorescence or electron microscopy.

There are several mouse models for colon cancer. A mouse model for FAP exists with heterozygous Apc mutations, while homozygous knockout of the Apc gene is embryonically lethal. Apc mutant mice develop several polyps in their small intestines that do not progress to adenocarcinoma, unlike the human characteristics of large intestinal polyps that advance to carcinomas (Moser et al., 1995). Moser developed the first mutant mouse model referred to as the multiple intestinal neoplasia (Min) model. Several other models have been developed, including the FabplCre; $Apc^{15lox/+}$ mouse model that has an extended lifespan and develops a number of cancerous polyps (Koratkar et al., 2002, 2004; Oikarinen et al., 2009; Robanus-Maandag et al., 2010). HNPCC or Lynch syndrome can also be modeled in mice with mutations to the DNA mismatch repair genes (MSH2, MLHJ, PMSJ, and PMS2). MSH2 knockdown in the villi

is one example (Kucherlapati et al., 2010). Other mouse models exist for the development of spontaneous CRC including those induced chemically with azoxymethane (AOM), dextran sulfate sodium (DSS), and 2-amino-1-methyl-6-phenylimidazol (4,5-b) (De Robertis et al., 2011; Karim and Huso, 2013). To measure tumor migration and invasion ability, xenografting is utilized, which involves the injection of cells or surgical transplantation of primary tissue into nude mice or SCID mice. Not all colon cancer cells are suitable for xenografting, but HCT-116 and HT-29 have great success. Other types of xenografts include patient-derived xenografts of liver metastases from CRC patients. This is a beneficial technique that prevents the genetic and epigenetic changes of cell culture (Fu et al., 1991; Rashidi et al., 2000). One limitation to using xenograft mouse models is the lack of an immune system. The immune system is vital to CRC development, as T-cell infiltration is a predictor for patient prognosis (Galon et al., 2006).

Future work includes determining the roles of the LXR and ER receptors on AKT signaling. To do this, p-AKT levels will be determined through western blotting in the presence of estrogen receptor and LXR agonists and antagonists. If changes occur with these compounds, then it would suggest that 27-OHC's action on p-AKT depends on ER or LXR. If no changes are seen, it would suggest an ER- and LXR-independent action. AKT inhibitors can also be used to determine if the decrease in cellular proliferation or increase in cellular migration is dependent on AKT signaling or if AKT signaling changes are an unrelated by-product of 27-OHC.

Conclusions

We found that in Caco2 and SW620 cells, 27-OHC leads to a decrease in cellular proliferation through both an MTT assay and CyQUANT® cell proliferation assay. This occurred at concentrations above physiological concentrations (10μM to 300μM). This decrease in cell proliferation is independent of cellular cytotoxicity and apoptotic cellular death. We next investigated the role of two common nuclear receptors that 27-OHC is a ligand for, ER and LXR. We found that upon treatment with ER and LXR agonists and antagonists, there were no significant changes in cellular proliferation, while the amount of ER and LXR isoforms were present. It appeared as though 27-OHC may be acting in an LXR- and ER-independent manner. To confirm this, we measured the amount of an LXR downstream gene (ABCG1) upon treatment with 27-OHC and an LXR agonist and antagonist. We found that 27-OHC didn't activate ABCG1 protein expression but the LXR agonist did activate the protein. This shows that 27-OHC does not act through the LXR pathway in these cells. Next, to determine if 27-OHC is activating ER, the localization of ER was examined using immunofluorescence. We found that treatment with 27-OHC doesn't lead to the nuclear localization of ER, which is instead found in the cytoplasm, showing that 27-OHC doesn't activate ER. Next, the cellular mechanisms of the decrease in cellular proliferation by 27-OHC were investigated. The role of IGF-1 and AKT signaling was investigated through western blotting, and we determined that there were no changes in IGF-1 expression upon treatment with 27-OHC. However, there was a significant decrease in the activated and phosphorylated form of AKT with 27-OHC treatment, suggesting that 27-OHC influences AKT signaling pathways. Overall, 27-OHC had strong effects on colon cancer cell proliferation.

CHAPTER III

27-OHC REGULATES SLFN12

Introduction and Rational

Schlafen proteins have an N-terminal AAA domain, which binds ATP/GTP and is involved in transcription. Then there is a "Slfn box" with an unknown function (Oh et al., 2011), followed by a C-terminal domain that functions in nuclear localization (Patel et al., 2009). There are three groups of Schlafen proteins with group I being between 37 and 42kDa, group II between 58-68kDa, and group II between 100-104kDa (Mavrommatis et al., 2013). Schlafen-12 is a class II Schlafen. Group III contains DNA/RNA helicase motifs that help with DNA/RNA metabolism (Brady et al., 2005). Schlafen is a group of proteins first discovered to play a role in thymocyte maturation (Schwarz et al., 1998). In addition to thymocyte maturation, Schlafen also plays a role in proliferation by inhibiting cyclin D1 and leading to cell cycle arrest (Brady et al., 2005). Another role is the regulation of differentiation of T cells and macrophages. Schlafen can also downregulate cancer cell migration and invasion and sensitizes cancer to chemotherapies (Kovalenko and Basson, 2014).

Schlafen proteins are involved in the immune system, especially during aspects of immune cell development such as T cell activation, macrophage differentiation, and monocyte maturation. Additionally, in humans, Schlafen proteins help to differentiate dendritic cells such that Schlafen 12 expression increases during differentiation of

dendritic cells (Puck et al., 2015). In mice, Schlafen 1 and 8 decrease T-cell proliferation (Neumann et al., 2008). SLFN 11 prevents HIV virus replication (Li et al., 2012). In cancer, Schlafens are upregulated by IFNα, which prevents tumor growth by inhibiting cell proliferation. Schlafens, specifically Schlafen 5, can regulate cancer cell invasion by mediating anchorage-independent growth in melanomas and matrix metalloproteinase to promote migration (Katsoulidis et al., 2010). Additionally, Schlafen-12 promotes prostate cancer cell differentiation (Kovalenko and Basson, 2014). In mice, Schlafen 2 and 3 downregulate metastasis and colony formation and increase differentiation. Schlafens are also involved in the increased susceptibility of cancer cells to targeted therapies, specifically SLFN12 improves tumor sensitivity to DNMDP in lung cancer cell lines (de Waal et al., 2016). Mouse SLFN3 enhances apoptosis and downregulates cancer stem cell markers such as CD44, CD133, and CD166 (Oh et al., 2011).

Schlafen 12 was first found to play a role in T-cell development and is involved in several immune system processes. In a study by Chaturvedi et al., they investigated whether an analogue of glucagon-like peptide 2 called Teduglutide impairs intestinal epithelial differentiation. The differentiation of epithelia is an important aspect of absorption and digestion. To test their hypothesis, they treated the colon cancer cell line, Caco2 with teduglutide and measured cell proliferation through an MTS assay along with bromodeoxyuridine. Additionally, RT-PCR was performed for several differentiation markers including SLFN12 and DPP-4. It was found that SLFN 12 was decreased by 61 percent with Teduglutide treatment, showing that Teduglutide treatment increased cellular proliferation and decreased epithelial differentiation (Chaturvedi and Basson, 2013).

Kovalenko et al. demonstrated that SLFN12 expression regulates prostate cell differentiation (Kovalenko et al, 2014). SLFN proteins have been shown to be involved in the differentiation of intestinal epithelial cells. Since differentiation is important for maintaining cells in a non-cancerous state, it was hypothesized that SLFN12 expression regulates prostate epithelial differentiation. To test this, LNCaP and PC-3 prostate cell lines were treated with an adenovirus that overexpressed SLFN12, and through RT-PCR it was determined that there was a significant decrease in PSA and an increase in DPP4. When SLFN12 was overexpressed, there was a decrease in proliferation. Finally, they showed that the changes in PSA and DPP4 were independent of ERK signaling. In conclusion, they found that SLFN12 modulates differentiation of prostate epithelial cells (Figure 17).

Differentiation is a process in which cells become more specialized and leads to changes in phenotype. In cancer, this process can be reversed and lead to dedifferentiation, a stage of cancer progression. Tumors with a high amount of differentiation, or a level similar to the native organ, have a better prognosis than those with a low amount of differentiation, where cells lose structural organization and all origins have been lost. During cancer, macrophages are activated and able to recognize surface receptors. Undifferentiated cancer cells have more surface irregularities and are more easily recognized by macrophages. Pathologists use these characteristics to grade cancer. Grade 1 is well differentiated and slow growing. Grade 2 involves moderately differentiated cancer cells that are growing faster than normal cells. Grade 3 is poorly differentiated and very aggressive.

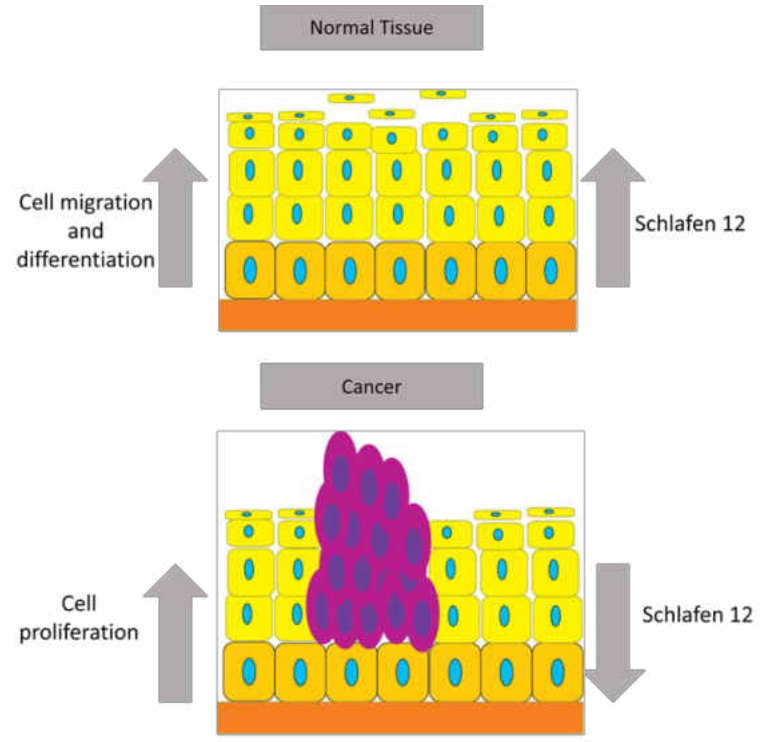

Figure 17. Cancer and SLFN12. In normal tissue, there is a balance between differentiation and proliferation in which there is an increase in SLFN protein. When cancer arises, there is an increase in cell proliferation and a decrease in cellular differentiation and SLFN12.

SLFN12 is a differentiation marker that controls the differentiation of prostate cells and is involved in the immune system. In cancer, the balance between differentiation and proliferation is vital. Therefore, understanding the role of SLFN12 in cancer is important to the regulation of cancer progression. 27-OHC is a molecule that has been shown to regulate proliferation in prostate, breast, and colon cells. Since SLFN12 regulates differentiation and proliferation of cells, we investigated whether the 27-OHC-mediated decrease in cellular proliferation is dependent on SLFN12 expression.

Methods and Materials

Materials

Caco2 cells (cat # HTB-37™), MTT Cell Proliferation Assay (cat # 30-1010K), and SW620 cells (cat # CCL-227™) (ATCC, Manassas, VA). DMEM media, Leibovitz's L-15 media, 100U/ml penicillin, 100ug/ml streptomycin, and 0.25ug/ml amphotericin (Life Technologies, Carlsbad, CA). FBS (Atlanta Biologicals, Flowery Branch, GA). GW3965 (cat # 2474), 27-hydroxycholesterol (cat # 3907), and estradiol (cat # 2824) (R&D systems from Minneapolis, MN). 6α-epoxycholesterol-3-sulfate (cat # C4136-000) (Steraloids, Newport, RI). CytoTox 96® non-radioactive cytotoxicity assay (cat # G1782) and DeadEnd Florometric TUNEL assay (cat # G3250) (Promega, Valencia, CA). Hard Set mounting medium with DAPI (cat # H-1500) (Vector Laboratories, Burlingame, CA). DNase (cat #AM2222), Alexa Fluor 594nm (cat #A11037), Halt™ proteases and phosphatase inhibitor cocktail (cat #78446) (Fisher Scientific, Hampton, NH). Immun-Blot® PVDF membrane for protein blotting (cat # 1620177), Clarity™ Western ECL substrate (BioRAD, Hercules, CA).

Cell Culture

Non-metastatic Caco2 cells were grown in DMEM media containing 10% FBS. Metastatic SW620 cells were grown in Leibovitz's L-15 containing 10% FBS. 100U/ml penicillin, 100ug/ml streptomycin, and 0.25ug/ml amphotericin were added. The cells were maintained at 5% CO_2 at 37°C and treated with 0, 0.5, 1, and 10μM of 27-hydroxycholesterol (27-OHC),

Western Blotting

The cells were harvested by a PBS wash and the addition of RIPA buffer with Halt™ proteases and a phosphatase inhibitor cocktail followed by spinning at 14,000g for 10 minutes at 4°C. The supernatant was then used for further processing. A Bradford assay using Bovine Serum Albumin (BSA) was used to quantify the protein concentrations of the lysates using a standard protocol. Between 10ug and 35ug of sample were separated using a 10 percent (15 percent for IGF-1) SDS-PAGE gel and transferred to a PVDF membrane. The membrane was incubated with antibodies (Abcam ab99198) and then exposed to Clarity™ Western ECL substrate. The blots were then visualized using the Omega Lum™ G imaging system (Aplegen). The bands were quantified through densitometry using ImageJ software.

RT-PCR

RNA was isolated after 8 and 24 hours of 27-OHC treatment using the QuickGene RNA cultured cell HC kit S (Autogen, Holliston, MA). Total RNA (1ug) was reverse transcribed into cDNA with qScript cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD). Real-time RT-PCR was performed on the cDNA. The data were quantified and expressed as fold-change compared to the control by using the $\Delta\Delta C_T$ method. Data are expressed as individual values with mean ± SEM and include determinations made with (n=3) and three technical replicates.

Results

Since SLFN12 is a protein involved in proliferation and differentiation of cancer cells, we wanted to determine the role of SLFN12 in 27-OHC-treated colon cancer cells. To accomplish this, Caco2 and SW620 cells were treated with varying amounts of

27-OHC (from 0μM to 10μM), and the amount of *SLFN12* was measured through RT-PCR (Figure 18). We found that there was no significant difference in the mRNA levels of *SLFN12* 24 hours after treatment with 27-OHC.

In addition to measuring mRNA levels of *SLFN12*, protein levels of SLFN12 were measured at 8 and 24 hours after treatment with various concentrations of 27-OHC. At 8 hours, we observed no change in SLFN12 levels in Caco2 cells, but we did observe a significant increase in SW620 cells at 0.5μM and 1μM (Figure 19). At 24 hours, there was no significant difference in SLFN12 protein levels after treatment with various concentrations of 27-OHC (Figure 20). These results demonstrate that 27-OHC leads to a transient increase in SLFN12 protein that disappears by 24 hours.

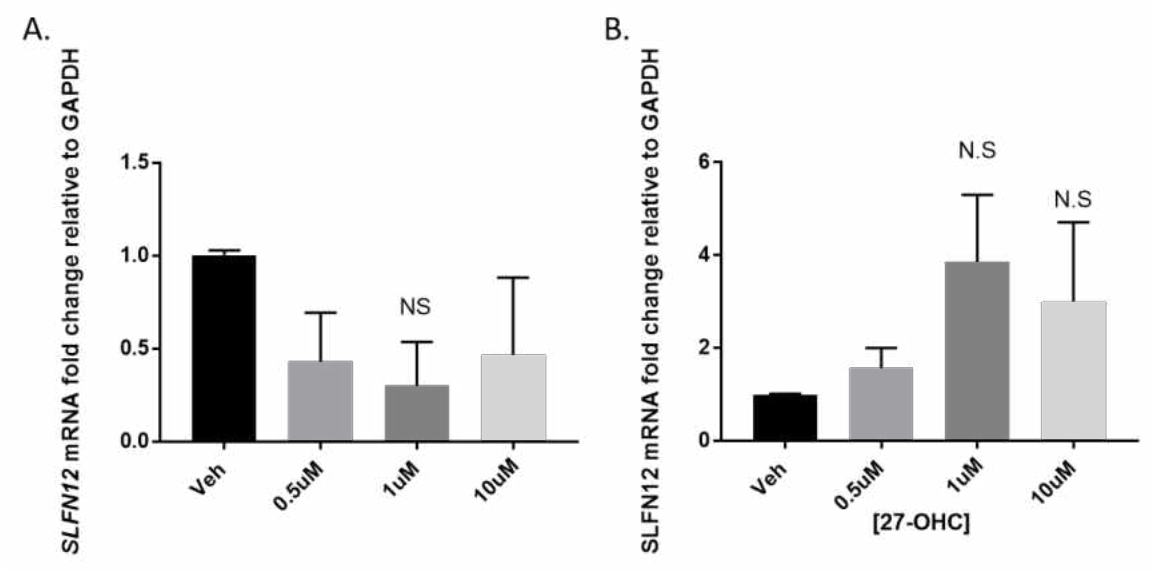

Figure 18. 27-OHC did not regulate SLFN12 at the mRNA level. SLFN12 mRNA levels were measured after treatment with different concentrations of 27-OHC in (A) Caco2 and (B) SW620 cells at 24 hours. All data is not signifcant.

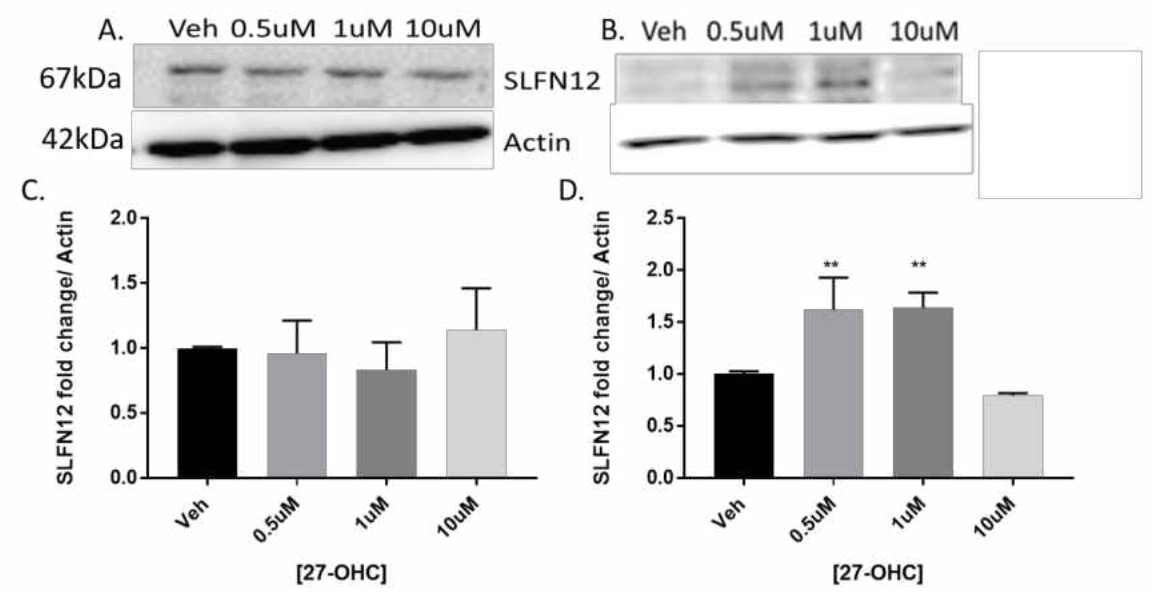

Figure 19. 27-OHC leads to an increase in SLFN12 at 8 hours. (A and B) are western blots of SLFN12 after 8 hours of treatment with 27-OHC in (A) Caco2 and (B) SW620 cells. (C and D) are the western blot quantification in (C) Caco2 and (D) SW620 cells. ** is p-value <0.01.

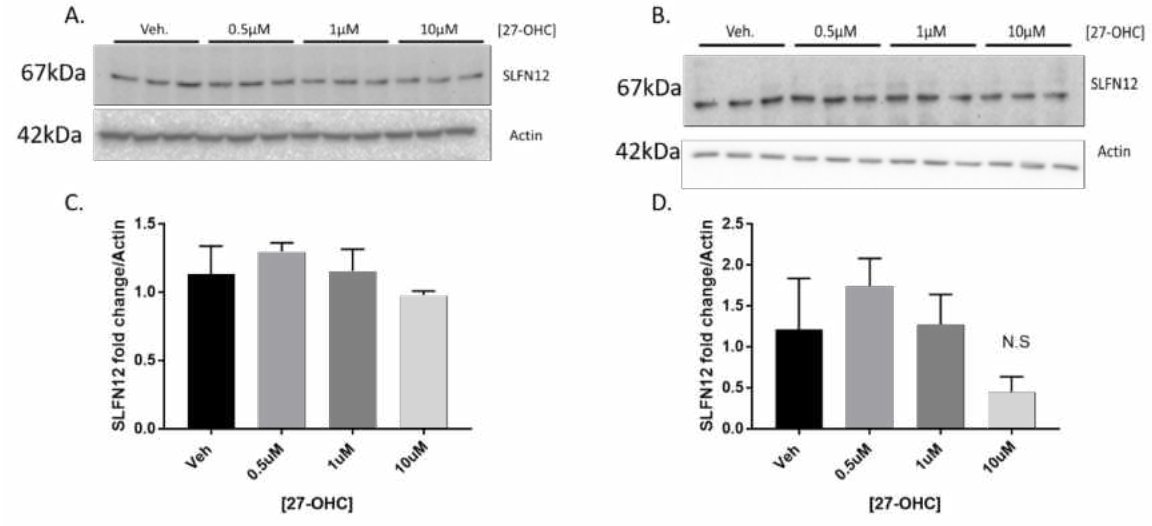

Figure 20. 27-OHC doesn't regulate SLFN-12 expression at 24 hours. Western blot analysis of Caco2 (A) and SW620 (B) with optical density analysis for Caco2 (C) and SW620 (D) showing no change in SLFN12 protein expression levels with increasing concentrations of 27-OHC. All data is not significant.

Discussion

The balance between proliferation and differentiation is vital in carcinogenesis and is regulated by SLFN12. Due to the fact that 27-OHC has been shown to play a role in proliferation, we investigated the impact of 27-OHC on SLFN12 expression. We found that there was no change in SLFN12 at the mRNA level after treatment with 27-OHC in both Caco2 and SW620 cells. At the protein level, there was a transient increase in SLFN12 expression after 8 hours of 27-OHC treatment but not at 24hours.

Since 27-OHC only increased SLFN12 expression after 8 hours of treatment in the metastatic SW620 cells and not in the Caco2 cells, our results suggest that in metastatic cancer there is more differentiation occurring and therefore an increase in SLFN12. A study by Eccles found that there was an inverse correlation between differentiation and metastatic potential in mouse models of mammary and squamous cell carcinomas (Eccles, 1983). In metastasis, the greatest proportion of tumor cells are differentiated with some metastasized tumors being more differentiated than the primary tumor (Brabletz, 2012). It would be interesting to see if treatment with 27-OHC at the same concentration that increases SLFN12 results in an increase in cellular migration (Ch 4) and if those migrating cells are also differentiated. This would provide a better understanding of the role of 27-OHC in colon cancer progression.

Since SLFN12 is a differentiation marker, the increase in SLFN12 by 27-OHC may indicate that 27-OHC is preventing the proliferation of these cells. It could be that 27-OHC is a protective molecule in carcinogenesis. Understanding the role of 27-OHC in the regulation of SLFN12 can lead to potential therapeutics for impeding cancer development and progression.

In the future, the role of the estrogen receptor and Liver-X-receptor in the SLFN12-mediated effects will be determined. Treatment of Caco2 and SW620 cells with the ER agonist estradiol or the ER antagonist ICI 182 780 followed by RT-PCR and western blotting for SLFN12 will help to determine if modulation of estrogen receptor regulates SLFN12, a new mechanism of regulation. Additionally, the role of LXR will be determined by using the LXR agonist GW3965 and the LXR antagonist ECHS, which will allow us to determine if 27-OHC is acting through LXR to regulate SLFN12 expression. In addition to measuring the level of SLFN12, other differentiation markers will be measured after 27-OHC treatment and treatment with the ER and LXR agonists and antagonists. Such markers will include DPP4.

Conclusions

We found that 27-OHC had no effect on *SLFN12* expression at the mRNA level at 24 hours. Also, at 24 hours there was no change in SLFN12 protein levels. However, at 8 hours there was an increase in SLFN12 protein levels in SW620 at 0.5μM and 1μM compared with vehicle control. This shows that 27-OHC has an effect on SLFN12 at an early timepoint that is lost at a later timepoint. It could be that 27-OHC signaling may play a role in differentiation, suggesting a new role for 27-OHC. This is also the first time, to our knowledge, that 27-OHC has been shown to regulate SLFN proteins, specifically SLFN12. This could open a new area of research focused on the role of oxysterols in cellular differentiation and modulation of cancer progression.

CHAPTER IV

27-OHC INCREASES CELLULAR MIGRATION

Introduction and Rational

A total of 90% of metastases result in cancer-related death; therefore, cellular migration from a primary tumor to another area of the body is a major health concern that should be studied. Cellular migration, which involves the movement of cells from one location to another, can occur during morphogenesis or development, wound healing, during an immune response, and metastasis. The process of migration has several stages including polarization, protrusion and adhesion, translocation of the cell body, and retraction of the rear. For cells to migrate, they need to be polarized or have a distinct front and rear, which is controlled by environmental signals that provide directionality. The front usually has more actin polymerization and adhesion to the substratum or surface that the cell attaches to when moving. The rear disassembles the adhesions. The front of the cell usually contains PIP3 and activated RAC and CDC42, while the back of the cell usually contains Rho GTPase and PTEN. The next step is protrusion or formation of membrane extensions of the leading edge, which are produced through actin polymerization. This involves expansion of the plasma membrane, formation of a backbone, and contact with the substratum. Next, adhesion to the substratum occurs through integrin receptors, whose activation links the substratum to the actin cytoskeleton, providing traction for the migration to occur. Some proteins involved in this

process include talin and α-actin. The cell body is translocated through myosin II and dynein. The rear is then retracted through adhesion and microtubule disassembly. (Figure 21). Problems with migration can lead to a variety of diseases such as autoimmune diseases, multiple sclerosis, and tumor metastasis (Matsuoka, 2014).

Metastasis is the result of a complicated set of molecular events. Major steps include tumor angiogenesis, disaggregation of the cell from the primary tumor, invasion through the basement membrane and extracellular matrix, invasion of the basement

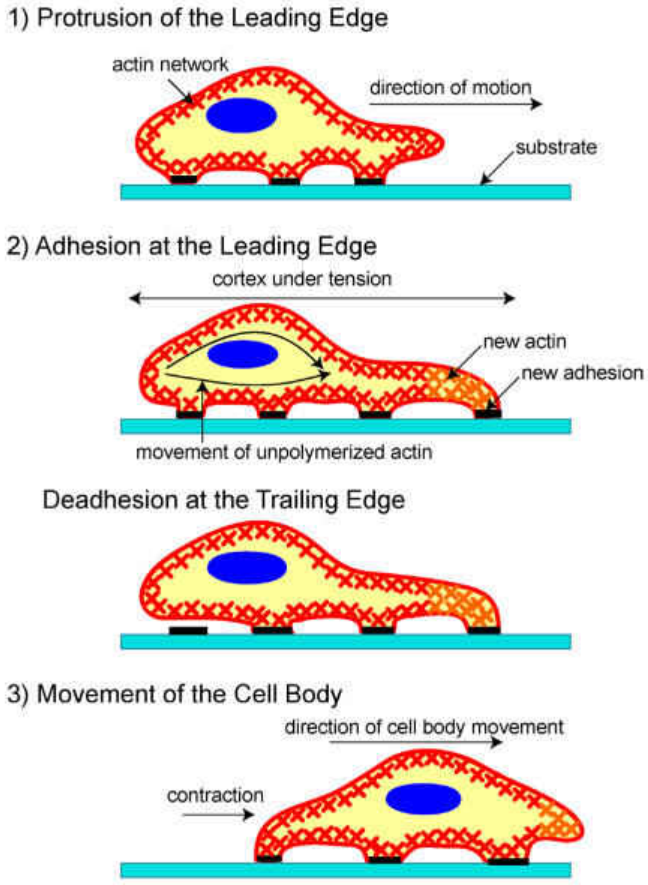

Figure 21. Cell migration. This process involves reorganization of the actin cytoskeleton, which is regulated by Rho GTPases. Reproduced with permission from Ananthakrishnan and Ehrlicher, 2007.

membrane of the endothelium of blood vessels, and invasion through endothelial cells and target organ tissues (extravasation). The first step involves invasion of cancer cells from the primary tumor site. The cells need to first migrate through the basement membrane, which is facilitated by the loss of E-cadherin (the epithelial marker that mediates junctions). Additionally, there is loss of tight junctions and cell polarity, which is controlled by several transcription factors including SNAIL, SLUG, TWIST, and ZEB1. The tumor cell is able to migrate through the basement membrane through proteolysis by matrix metalloproteinases (MMPs), which also helps cells to proliferate by secretion of growth factors. After passing through the basement membrane, the tumor cells next enter the stroma where they encounter fibroblasts, adipocytes, macrophages, and mesenchymal stem cells. Interactions with the stroma increase the aggressiveness of the invasion through several signaling pathways such as IL-6 and EGFR signaling. The tumor cells then enter into blood vessels or the lymphatic system. This process involves cytokine transforming growth factor β (TGFβ), epidermal growth factor (EGF), and colony stimulating factor 1 (CSF-1). New blood vessel formation is also important and relies on vascular endothelial growth factor (VEGF). Once inside the blood vessels, tumor cells begin to circulate. During circulation, the tumor cell is prone to cell death due to lack of attachment to the extracellular matrix and the innate immune system. However, metabolic changes promote the survival of these tumor cells. Next, the tumor cells settle within microvessels of different organs, forming a microcolony. The cells then extravasate through extraction of MMPs (MMP1, 2, 3, and 10), and a micrometastases environment is formed, leading to colonization of tumor cells at a distinct site (Ganguly et al., 2013)(Figure 22)

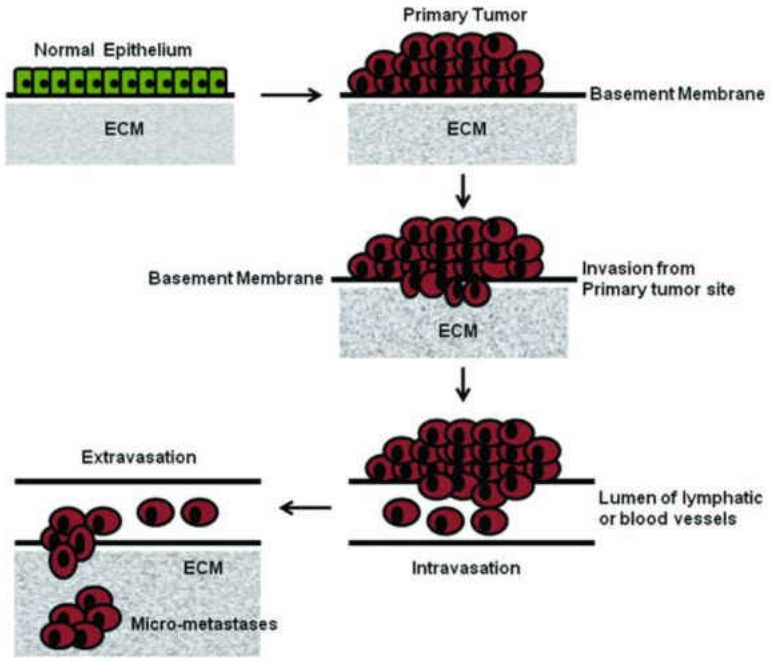

Figure 22. Vital events of the metastatic cascade. The primary tumor invades through the ECM, then through the lympahatic or blood vessels to form micro-metastasises. Reproduced with permission with permission from Ganguly et al., 2013. Copyright 2012 Landes Bioscience.

An important component of cellular migration is the loss of epithelial markers and the gain of mesenchymal markers. Some examples include the loss of E-cadherin and the gain of N-cadherin. Cadherins are glycoproteins involved in cell-cell adhesion, and they are calcium-dependent. There are five subfamilies of cadherins that include classical cadherins type I, type II, desmosomal cadherins, protocadherins, and cadherin-related molecules. E-cadherin is a type I cadherin that is localized on the surface of epithelial cells where cells make cell-to-cell contact. E-cadherin is 120kDa long with an extracellular domain, a single transmembrane region, and a short cytoplasmic domain. The cytoplasmic domain interacts with the actin cytoskeleton through α, β, and γ catenins and functions to regulate cell-to-cell binding. The extracellular domain has pockets for

calcium binding. Epithelial cells have strong cell-to-cell interactions through tight junctions, adherent junctions, and desmosomes. E-cadherin interacts with epithelial growth factor receptor (EGFR) and is involved in WNT signaling (van Roy and Berx, 2008). N-cadherin allows cells to be motile and uses FGFR to increase a cell's ability to migrate through the ECM and blood vessels. It recruits PI3K, activating AKT, which leads to inactivation of Bad, and also decreases cell proliferation by increasing the amount of time in G2/M phase (Derycke and Bracke, 2004).

Raza et al. showed that 27-OHC significantly reduced invasion in the LNCaP and PC3 prostate cancer cell lines but not in normal RWPE-1 cells (Raza et al., 2017). Additionally, in smooth muscle cells that mimic atherosclerosis, they found that treatment with the oxysterols 7-ketocholesterol or 27-hydroxycholesterol led to an inhibition of migration of smooth muscle cells. However, treatment with cholesterol did not reduce migration (Oyama et al., 2002). Recent work by Shen et al. showed that 27-OHC upregulates cellular invasion and migration in breast cancer cells and underwent epithelial to mesenchymal transition (EMT). This occurs through the signal transducer and activator of transcription 3 (STAT-3), which upregulates matrix metalloproteinase 9. They showed that upon treatment with 27-OHC for 72 hours, there was an increase in cellular migration and invasion. After 48 hours of treatment with 27-OHC, there was an increase in mesenchymal markers such as vimentin and ZEB-1 and a decrease in the epithelial marker E-cadherin (Shen et al., 2017). Additionally, Sierralta et al. showed that 27-OHC decreases E-cadherin and β-catenin levels and leads to the loss of adherent junctions, demonstrating that EMT occurs at 48 and 72 hours. Since 27-OHC has been

shown to be involved in cellular migration and invasion, we investigated the role of 27-OHC in cellular migration in two colon cancer cell lines.

Methods

Materials

Caco2 cells (cat # HTB-37™), MTT Cell Proliferation Assay (cat # 30-1010K), and SW620 cells (cat # CCL-227™) (ATCC, Manassas, VA). DMEM media, Leibovitz's L-15 media, 100U/ml penicillin, 100ug/ml streptomycin, and 0.25ug/ml amphotericin (Life Technologies, Carlsbad, CA). FBS (Atlanta Biologicals, Flowery Branch, GA). GW3965 (cat # 2474), 27-hydroxycholesterol (cat # 3907)(R&D systems from Minneapolis, MN). Halt™ proteases and phosphatase inhibitor cocktail (cat #78446) (Fisher Scientific, Hampton, NH). Immun-Blot® PVDF membrane for protein blotting (cat # 1620177), Clarity™ Western ECL substrate (BioRAD, Hercules, CA).

Cell Culture

Non-metastatic Caco2 cells were grown in DMEM media containing 10% FBS. Metastatic SW620 cells were grown in Leibovitz's L-15 containing 10% FBS. 100U/ml penicillin, 100ug/ml streptomycin, and 0.25ug/ml amphotericin were added. Cells were maintained at 5% CO_2 at 37°C and treated with 0, 0.5, 1, and 10µM of 27-hydroxycholesterol (27-OHC) after reaching 85 percent confluency.

Western Blotting

Cells were harvested by a PBS wash and the addition of RIPA buffer with Halt™ proteases and a phosphatase inhibitor cocktail followed by spinning at 14,000g for 10 minutes at 4°C. The supernatant was then used for further processing. A Braford assay was performed using Bovine Serum Albumin (BSA) to quantify the protein

concentrations of the lysates using a standard protocol. Between 10ug and 35ug of sample was separated using 10 percent (15 percent for IGF-1) SDS-PAGE gels and transferred to a PVDF membrane. The membrane was incubated with an E-cadherin antibody (Cell signaling 3195S) and an N-cadherin antibody (cell signaling 13116S). The membrane was then exposed to Clarity™ Western ECL substrate. The blots were then visualized using an Omega Lum™ G imaging system (Aplegen). The bands were quantified through densitometry using ImageJ software.

Scratch Assay

A total of $1x10^6$ cells were added per well and incubated overnight. The next morning a p-200 tip was used to create a scratch on the cell surface. The cells were then observed and photographed every 4 hours for 48 hours. The amount of closure between the scratch was determined using ImageJ software, and fold change of migration was determined.

Results

27-OHC Increases Cellular Migration

To measure the amount of migration occurring, a scratch assay was performed in the presence or absence of 27-OHC in two colon cancer cell lines: Caco2 (nonmetastatic) and SW620 (metastatic). We observed a significant increase in the amount of cellular migration with 27-OHC treatment relative to vehicle control (Figure 23 A-D). This result demonstrates that 27-OHC increases cellular migration in colon cancer cells.

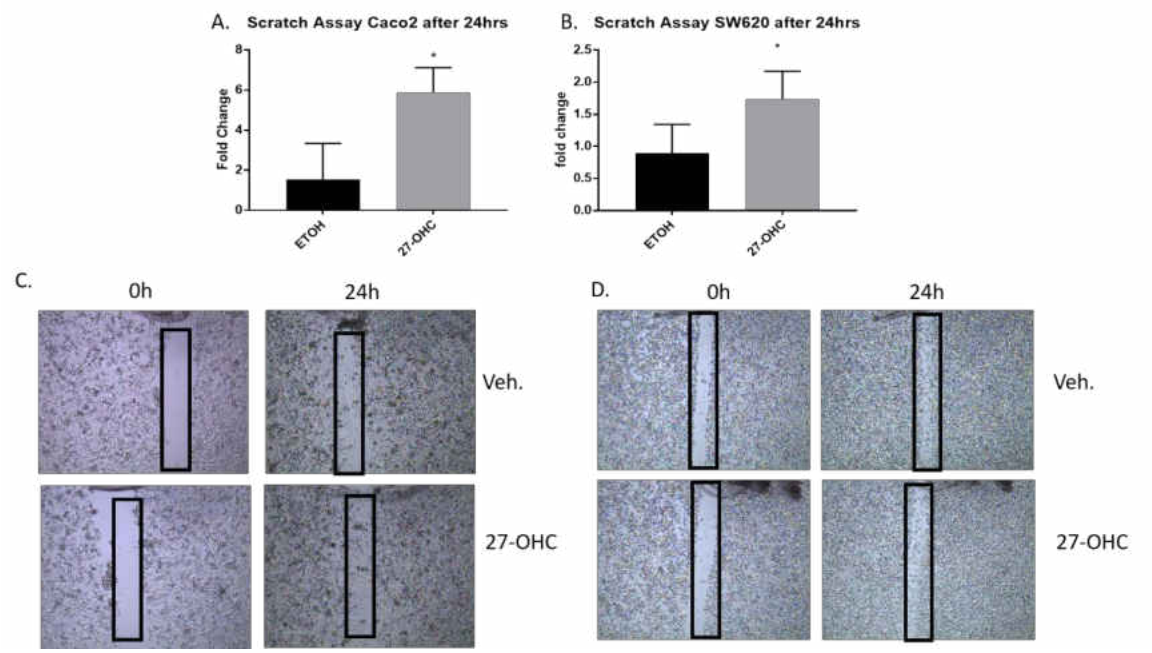

Figure 23. 27-OHC increases cell migration via scratch assay. Caco2 (A and C) and SW620 (B and D) were subjected to a scratch assay to measure cell migration. A and B are quantifications of three scratch assays while C and D are representative examples of scratch assay closure after 24 hours in the presence or absence of 27-OHC. * ip-value <0.01.

27-OHC Decreases E-Cadherin with no Change in N-Cadherin

Since 27-OHC led to an increase in cellular migration, we assessed whether there were changes in epithelial and mesenchymal markers to determine if the cells were undergoing an epithelial to mesenchymal transition. After treatment with 27-OHC, we observed a significant decrease in the epithelial marker E-cadherin (Figure 24) in both cell lines. However, treatment with 27-OHC did not significantly alter the amount of N-cadherin after 24 hours (Figure 25). This suggests that after 27-OHC treatment, the cells begin to undergo a loss of epithelial markers and EMT. However, complete EMT was not demonstrated because there was no concurrent gain of mesenchymal markers.

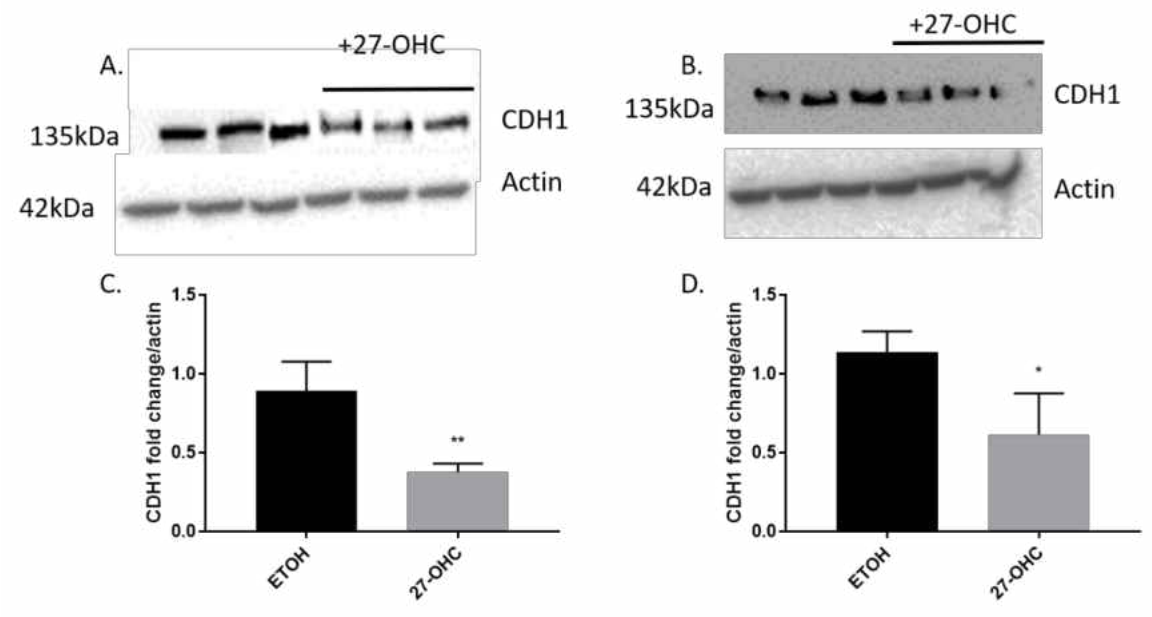

Figure 24. 27-OHC decreases E-cadherin. Western blot of E-cadherin in Caco2 (A and C) and SW620 (B and D) cells after 24 hours of 27-OHC treatment with western blot quantification in C and D compared to vehicle control. * p-value <0.05 and ** p-value <0.01.

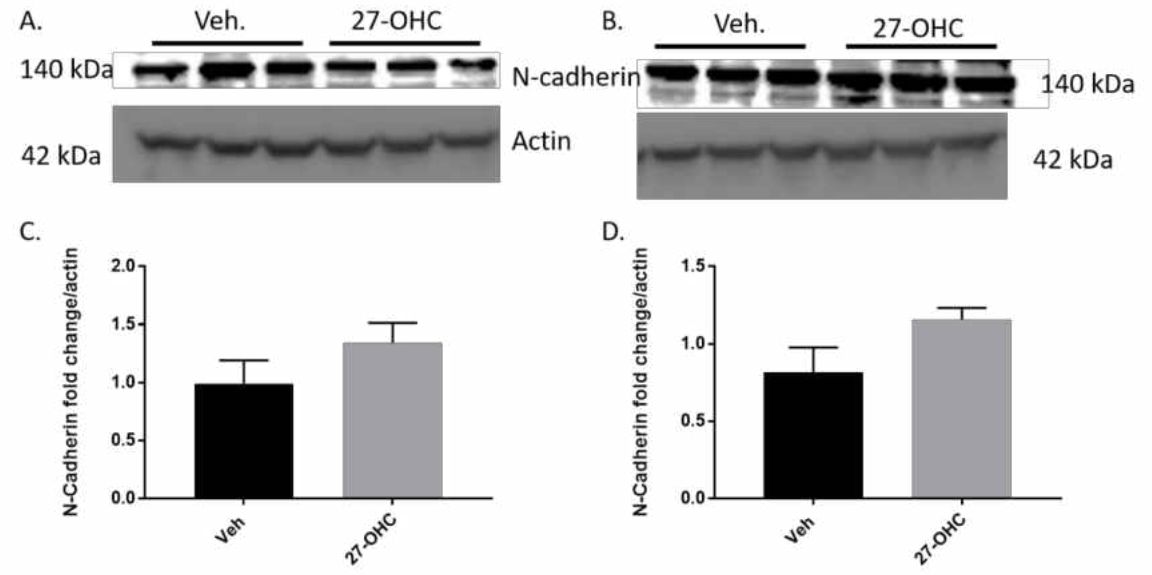

Figure 25. 27-OHC doesn't affect N-cadherin after 24 hours. Western blot of N-cadherin in Caco2 (A and C) and SW620 (B and D) cells after 24 hours of 27-OHC treatment with western blot quantification in C and D compared to vehicle control. All data was not significant.

Discussion

In summary, I have demonstrated that treating colon cancer cell lines with 27-OHC led to an increase in cellular migration that was accompanied by a loss of E-cadherin without any changes to N-cadherin. These results indicate that the cells underwent partial but not a complete EMT. A longer duration of 27-OHC treatment may be needed to complete the EMT process.

I observed an increase in cellular migration and a loss of E-cadherin, which was similar to previous results reported by Shen et al. (Shen et al., 2017). Shen et al. also showed that there was an increase in mesenchymal markers. However, this wasn't observed in my experiments. This could be due to differences in treatment length. Shen et al observed changes after 48 and 72 hours. I, however, only examined N-cadherin after 24 hours. It is possible that 24 hours is not enough time to undergo complete EMT and therefore I didn't see changes in N-cadherin. This could also be due to differences in cell line specificity. In the future, N-cadherin could be measured after a longer duration of treatment with 27-OHC, such as 48 or 72 hours instead of 24 hours.

Other oxysterols also play a role in cellular migration. In smooth muscle cells, the roles of 7-ketocholesterol and cholesterol 5α,6α (α-epoxide) in cellular migration and proliferation have been investigated. These oxysterols increased smooth muscle cell migration and increased proliferation. It was determined that the migration changes occurred through changes in MMPs, EGFR, and PI3K, followed by activation of AKT (Liao et al., 2010). Cholestane-3β, 5α, 6β-triol (triol) resulted in a reduction in migration in DU-145 and PC3 prostate cancer cell lines with an increase in E-cadherin but a loss of N-cadherin, vimentin, and slug. There were also changes in the localization of β-actin and

α-tubulin (Lin et al., 2013). In lung adenocarcinoma, 25-hydroxycholesterol has been shown to promote cellular migration and invasion in an LXR-dependent manner (Chen et al., 2017).

The estrogen receptor may play a role in migration, with the ERβ inhibitor PHTPP leading to changes in invasion in the normal prostate cell line RWPE-1 and the prostate cancer cell line LNCaP (Raza et al., 2017). Additionally, LXR plays a role in 25-OHC-induced migration and invasion (Chen et al., 2017). LXR activation through T0901317 also inhibits cellular invasion in the prostate cancer cell line PC3, and LXRα activation reduces β-catenin expression (Youlin et al., 2017).

Studies to determine the molecular mechanism of 27-OHC-induced cellular migration and EMT have yet to be completed. This would be an area of great interest because if we can determine how 27-OHC results in cellular migration, then we could prevent it pharmaceutically and improve the overall health of cancer patients. To determine the molecular mechanism, it is important to determine the role of 27-OHC's nuclear receptor targets LXR and ER. Future directions include measuring cellular migration in the presence or absence of ER and LXR agonist and antagonists. This would help determine if the effects of 27-OHC on cellular migration are dependent on ER or LXR activation. If treatment with the LXR and ER agonist and antagonist results in changes in cellular migration, then that would suggest that the 27-OHC-induced changes in cellular migration are due to activation of LXR or ER. If no changes in migration are observed, it would suggest that 27-OHC's effect is LXR- and ER-independent and may be using another mechanism. Also, changes in EMT markers such as E- and N-cadherin will be determined in the presence or absence of ER and LXR agonists and antagonists.

Additionally, I would measure E- and N-cadherin after a longer duration of 27-OHC treatment, such as 72 hours, to give the cells a longer time to respond to the treatment to complete an EMT.

Another important aspect of EMT and cancer progression is cellular invasion, or migration of cells through a 3-D matrix. Cells need to invade the extracellular matrix (ECM) in order to invade a new tissue. Cells can migrate as a single cell (mesenchymal state) or in sheets (epithelial). One assay to measure cellular invasion is a transwell cell migration and invasion assay. A layer of extracellular matrix or Matrigel is added on top of a transwell, and the cells are allowed to migrate towards a chemoattractant. Then, the amount of cells is measured through microscopy, or if a fluorescent dye is used, through a plate reader. This is a relatively quick and efficient way to measure cellular invasion. Some limitations include that preliminary experiments are required to optimize the conditions of invasion. In order to determine whether 27-OHC affects cellular invasion, 27-OHC will be added to the cells and the amount of cellular invasion will be measured. Additionally, the role of ER and LXR agonists and antagonists on cellular invasion will be measured to determine if the effect on cellular invasion is dependent on ER and LXR activation.

Conclusion

Cellular migration is an important part of tumor metastasis, a marker of an aggressive cancer phenotype. To measure cellular migration, a scratch assay was performed. We determined the role of 27-OHC by treating both Caco2 and SW620 cells with it, followed by a scratch assay. We found that 27-OHC treatment leads to an increase in migration relative to vehicle control in both cell lines. An additional marker of

cellular migration is the loss of the epithelial marker E-cadherin and the gain of the mesenchymal marker N-cadherin. We found that there was a significant decrease in E-cadherin in Caco2 and SW620 cells with no changes in N-cadherin. This suggests that the 27-OHC-treated cells are beginning to undergo epithelial to mesenchymal transition with the loss of E-cadherin. Longer exposure to 27-OHC may result in a gain of N-cadherin. More work needs to be done to determine the molecular mechanisms of 27-OHC's effects on cellular migration and whether LXR and ER are involved. To our knowledge, this is the first study to demonstrate that 27-OHC regulates cellular migration in colon cancer.

CHAPTER V

OVERALL SUMMARY AND CONCLUSIONS

Summary

In summary, we found that 27-OHC has a wide variety of effects in colon cancer cell lines such as Caco2 and the metastatic cancer cell line SW620. We began by investigating the role of 27-OHC in cellular proliferation and found that 27-OHC decreased proliferation and the effects were independent of cellular cytotoxicity and apoptotic cellular death. In addition, 27-OHC induced proliferation and the effects were independent of its nuclear receptors liver-x-receptor and estrogen receptor. We found that 27-OHC treatment led to a decrease in the phosphorylated and active forms of AKT in Caco2 cells but not in SW620 cells, suggesting that 27-OHC may be regulating cellular proliferation through AKT signaling. We also found that 27-OHC induced a transient increase in the differentiation marker SLFN12 at 8 hours that was lost after 24 hours of 27-OHC treatment in SW620 cells but not Caco2 cells. We also measured cellular migration and found that treatment with 27-OHC led to an increase in cellular migration after 24 hours. In addition, we investigated epithelial to mesenchymal markers and found that there was a loss of E-cadherin with no change in N-cadherin after 27-OHC treatment.

Long Term Implications

Overall, our study shows that 27-OHC decreases cellular proliferation with a subsequent increase in cellular migration. This can be explained by the fact that cells cannot proliferate and migrate at the same time and must stop proliferating in order to

migrate. Our results may suggest that 27-OHC treatment leads to cellular migration in colon cancer. Therefore, it is important that the levels of 27-OHC remain in check during colon cancer development to prevent the spread of cancer to other areas in the body. This can be accomplished through inhibition of CYP27A1, which catalyzes the hydroxylation of cholesterol to form 27-OHC. If there are lower levels of 27-OHC, then the prognosis of colon cancer can be improved (Figure 26).

We demonstrated that 27-OHC leads to a decrease in cellular proliferation. It would be interesting to know where in the cell cycle these abnormalities are occurring. To study this, propidium iodide would be used followed by flow cytometry to determine

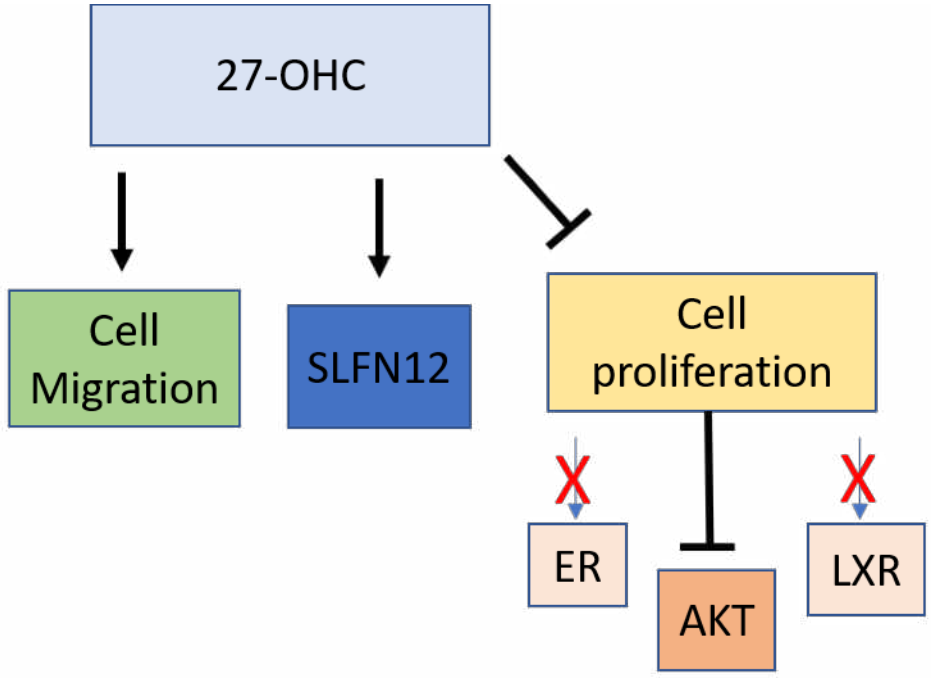

Figure 26. Overall Conclusion. 27-OHC leads to a decrease in cellular proliferation that is independent of ER and LXR activation while also increasing SLFN12 levels and cellular migration.

the number of cells in each phase of the cell cycle, giving an indication of problems with the cell cycle. Once the phase of the cell cycle has been identified, then certain cyclins and CDKs can be measured along with different cell cycle regulators. This will allow for the molecular mechanisms of 27-OHC to be determined and for therapeutic targets to be developed.

We showed that 27-OHC leads to cellular proliferation independently of activation of the estrogen receptor or liver-x-receptor. This suggests that 27-OHC is acting through some other mechanism. 27-OHC could be acting through some of the proteins it binds to including INSIG, SREBP, NPC, and other members of the oxysterol-binding protein family (OSBPs). Specifically, target compounds of OSBPs lead to inhibition of cellular growth and anti-proliferation effects (Du et al., 2017). However, it may be possible that a novel or uncharacterized pathway led to the changes in proliferation. More experiments, such as siRNA knockdown of certain proteins, need to be performed to characterize the system.

I also found that AKT is downregulated by 27-OHC. The exact mechanism of action for 27-OHC in the AKT pathway is unknown and should be investigated to identify potential therapeutic products. 27-OHC could be affecting proteins that are upstream of p-AKT, such as PI3L, PIP3, and PDK1. To determine the impact of reductions in 27-OHC on p-AKT, a vector that overexpresses AKT will be utilized. I will then treat cells with 27-OHC and measure the amount of cellular proliferation to see if I can rescue the amount of proliferation through an MTT assay. Other upstream targets of AKT will be examined as well. These experiments will shed light on the molecular

mechanisms of the 27-OHC-mediated control of AKT, leading to possible therapeutic targets.

27-OHC also led to a transient increase in SLFN12 expression, a colon differentiation marker. This could indicate that 27-OHC can lead to changes in differentiation. siRNA against SLFN12 followed by treatment with 27-OHC can be used to determine the role of 27-OHC in differentiation and proliferation, and other differentiation markers will also be measured. Additionally, overexpression of SLFN12 can be expressed using an expression vector. Both sets of experiments can help determine how 27-OHC influences the role of SLFN12 in colon cancer progression.

27-OHC increased cellular migration through a loss of E-cadherin with no gain of N-cadherin, indicating early signs of EMT. In addition to investigating migration, we will investigate cellular invasion, another important part of tumor movement to another area of the body. Stopping cellular migration and invasion is an important therapeutic target. Therefore, understanding the molecular mechanism of 27-OHC-induced migration is important. To do this, the involvement of LXR and ER in cellular migration will be investigated. Specifically, ER and LXR agonists and antagonists will be used to measure cellular migration and invasion to investigate the mechanisms of 27-OHC-induced migration and EMT.

For my future studies, I would use an inducible colon cancer model to replicate the majority of CRC cases through chemical treatments such as DSS. I would treat mice with DSS and also 27-OHC and measure the amount of time it takes to develop CRC and the tumor volume. I would also measure the amount of tumor metastasis in the liver and lungs. Since 27-OHC leads to an increase in cellular migration, I hypothesize that

27-OHC would increase the tumor burden in the lungs and liver relative to vehicle control. In a xenograft model, I would expect 27-OHC to increase the amount of metastasis. If I observe changes with 27-OHC, I would then also examine the role of the ER and LXR receptors in tumor metastasis to determine whether or not it is dependent on ER or LXR.

Since 27-OHC led to an increase in cellular migration along with a decrease in cellular proliferation, there appears to be a more aggressive phenotype. This more aggressive phenotype can be prevented if 27-OHC is not produced. This can occur if the enzyme that makes 27-OHC, CYP27A1, is inhibited. Several different approaches can be used to target the expression of CYP27A1, such as siRNA and CRISPR. Other techniques include targeting CYP27A1 with drugs.

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