MODULATION OF NUCLEAR RECEPTOR FUNCTIONS IN BREAST AND PANCREATIC CANCERS

A Dissertation Presented to

the Faculty of the Department of Biology and Biochemistry

University of Houston

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

By

Nicholes R Candelaria

May 2015

MODULATION OF NUCLEAR RECEPTOR FUNCTIONS IN BREAST AND PANCREATIC CANCERS

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Nicholes R Candelaria

APPROVED:

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Dr. Chin-Yo Lin, Chairman

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Dr. Daniel Frigo

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Dr. Kevin Phillips
The Methodist Hospital Research Institute

_____

Dr. Cecilia Williams

_____

Dr. Dan Wells, Dean, College of Natural Sciences and Mathematics

ACKNOWLEDGEMENTS

I am so incredibly grateful to the people in my life, as I wouldn't be anyone without their help. My mom is my greatest support and my best friend. She knows me for all my faults and virtues, and is an incredibly fair-minded, compassionate, and beautiful human being. My mom cuts wood, tends daily to cows and horses, and takes care of all her kids. She loves to garden. She also happens to be the most naturally intelligent person I know. Despite our rural upbringing, she recognized early on the value of an education in a location that isn't known for their thinkers. She listens to our troubles, and is genuinely excited when we succeed. My mother knows first hand the toll cancers take on individuals and their loved ones, as she has carried a loved one through cancer. My grandmother Faye was given no hope of survival. Faye stayed with us as she battled her metastatic ovarian cancer, which is probably the most terrifying and cruel way to die. I miss her very much and wish she were still here.

I would also like to thank my father, Larry, who supported our family all these years. I sincerely miss his enchiladas. I would also like to thank my siblings, Donnie, Melissa, and Lindsay. Donnie is my older brother and shows so much love and concern for me. I love his girlfriend Shanna as a third sister. My older sister Melissa is a brilliant cook and a gifted explosives engineer with two amazing kids and a husband. Lindsay, my younger sister, is a very trusting and kindhearted person, and is always giving others the benefit of the doubt. I'm so proud of her. Lastly, I would like to thank my boyfriend, Chaseton, who puts up with me on a daily basis. He is a gifted comedian and we're always laughing about something. I'm lucky to have found someone that I have so much in common with.

I am incredibly grateful to my committee for serving in such a charitable way. I am grateful to Dr. Williams for assisting in the midst of a sabbatical in Sweden. She has been most kind and supportive, and has helped us identify new mechanisms of alcohol in breast cancer. Tack för allt. I appreciate Dr. Frigo for his mechanistic insights and his sense of humor. He has given me great advice that I will use in the coming months and years. I am also in the debt of Dr.

Phillips, who comes down from Methodist Hospital to serve on my committee. He has been nothing but supportive and has made suggestions that were of use in the alcohol studies.

I would also like to thank my group members, first Sridevi, who worked with me on the pancreatic cancer project. Sridevi cares for people in ways the average scientist doesn't. This project wouldn't have been nearly as successful if Sridevi wasn't involved. Husna has a very logical thought process, and a strong ethical center that I will miss. Helena was always very calm and collected, and handles work very professionally. I will miss my coffee runs with Marisa Simon, who bakes me gluten-free goods and has always treated me with so much respect. I would also like to thank faculty, staff, and graduate students in the Center for Nuclear Receptors and Cell Signaling.

Lastly, I would like to thank Dr. Chin-Yo Lin, who despite his teasing is incredibly patient, kind, and fair. I still remember taking Dr. Lin's genomics class back at BYU. I really connected with his lecture style, and I found him logical, engaging, and funny. I then found out Dr. Lin worked on breast cancer. He agreed to let me do research in his lab at BYU. I was not the most gifted of lab technicians. Dr. Lin hung with me as I figured out how to carry out scientific experiments. He gave me a chance to improve my laboratory work, and I am so much better now because of it. I don't imagine that anyone else at BYU would've taken a student with a desire to do research, but limited laboratory talent such as myself. Put short, my time with Dr. Lin has had a profoundly positive impact on my life, and if I could return all the kindness he's shown me these seven years, he would have a *Cell* or *Nature* publication. I have many debts, which I hope to repay in the coming months and years.

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ABSTRACT

Alcohol Regulates Genes that are Associated with Response to Endocrine Therapy and Attenuates the Actions of Tamoxifen in Breast Cancer Cells

Pancreatic ductal adenocarcinoma (PDAC) is difficult to detect early and is often resistant to standard chemotherapeutic options, contributing to extremely poor disease outcomes. Members of the nuclear receptor superfamily carry out essential biological functions such as hormone signaling and are successfully targeted in the treatment of endocrine-related malignancies. Liver X receptors (LXRs) are nuclear receptors that regulate cholesterol homeostasis, lipid metabolism, and inflammation. Intriguingly, LXR agonists exhibit antiproliferative activity in diverse types of cancer cells. In this study, LXR agonist treatments disrupted proliferation, cell-cycle progression, and colony-formation of PDAC cells. At the molecular level, treatments downregulated expression of proteins involved in cell cycle progression and growth factor signaling. Microarray experiments further revealed changes in expression profiles of multiple gene networks involved in biological processes and pathways essential for cell growth and proliferation following LXR activation. These results establish the antiproliferative effects of LXR agonists and potential mechanisms of action in PDAC cells and provide evidence for their potential application in the prevention and treatment of PDAC.

The Antiproliferative Properties and Mechanisms of LXR Ligands on Pancreatic Ductal Adenocarcinoma Cells

Hereditary, hormonal, and behavioral factors contribute to the development of breast cancer. One modifiable behavioral factor, alcohol consumption, is linked to breast cancer risk. In this study we characterized molecular mechanisms of action of alcohol in estrogen receptor (ER)-positive breast cancer cells. Treatments with alcohol promoted cell proliferation, increased growth factor signaling, and up-regulated the transcription of the ER target gene *GREB1*. Microarray analysis following alcohol treatment identified a large number of alcohol-responsive genes, which were strongly associated with clinical outcomes in patients who received endocrine therapy.

Correspondingly, alcohol treatment attenuated the anti-proliferative effects of the endocrine therapeutic drug tamoxifen in ER-positive breast cancer cells. To determine the contribution and functions of responsive genes, their differential expression in tumors were assessed between outcome groups. The proto-oncogene *BRAF* was identified as a novel alcohol- and estrogen-induced gene that showed higher expression in patients with poor outcomes. Knock-down of *BRAF*, moreover, prevented the proliferation of breast cancer cells. These findings not only highlight the mechanistic basis of the effects of alcohol on breast cancer cells and increased risks for disease incidents and recurrence, but may facilitate the discovery and characterization of novel oncogenic pathways and markers in breast cancer research and therapeutics.

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Published Manuscripts included in this dissertation:

Candelaria, N. R., Addanki, S., Zheng, J., Nguyen-Vu, T., Karaboga, H., Dey, P., Gabbi, C., Vedin, L.L., Liu, K., Wu, W., Jonsson, P. K., Lin, J. Z., Su, F., Reddy-Bollu, L., Hodges, S. E., McElhany, A. L., Issazadeh, M. A., Fisher, W. E., Ittmann, M. M., Steffensen, K. R., Gustafsson, J. Å, & Lin, C. Y. (2014). Antiproliferative Effects and Mechanisms of Liver X Receptor Ligands in Pancreatic Ductal Adenocarcinoma Cells. *PloS One*, *9*(9), e106289.

Nguyen-Vu, T., Vedin, L. L., Liu, K., Jonsson, P., Lin, J. Z., Candelaria, N. R., Candelaria, L. P., Addanki, S., Williams, C., Gustafsson, J. Å, Steffensen, K. R., & Lin, C. Y. (2013). Liver X receptor ligands disrupt breast cancer cell proliferation through an E2F-mediated mechanism. *Breast Cancer Research*, *15*, R51.

*Candelaria, N. R., Liu, K., & Lin, C. Y. (2013). Estrogen receptor alpha: Molecular mechanisms and emerging insights. *Journal of Cellular Biochemistry*, *114*(10), 2203-2208.

Manuscripts in revision included in this dissertation:

Candelaria, N.R., Weldon, R., Muthusamy, S., Nguyen-Vu, T., Addanki, S., Folly-Kosi, H., Karaboga, H., Blessing, A., Reddy-Bollu, L., & Lin, C. Y. (2015). Alcohol attenuates tamoxifen response in ER+ breast cancer cell lines and regulates genes that are tied to patient survival and response to therapy. *PloS One.*

**Materials in this publication are used in chapter 4.*

1. Introduction

1.1 The Nuclear Receptor Superfamily

1.1.1 Nuclear Receptor Ligands and Mechanisms

Nuclear receptors (NRs) encompass a large family of transcription factors that sense biochemical changes within metazoans and alter physiological responses to changing internal and external conditions[1,2]. Members of the nuclear receptor superfamily evolved from a single ancestral NR to the current 48 found in humans [1,3,4]. These proteins regulate metabolic, developmental, and homeostatic cellular processes in response to dynamic levels of lipophilic signaling molecules (ligands), which can act as activators, repressors, or modulators by binding and altering NR structure and function [3]. Structurally, NR signaling ligands are thyroid, retinoid, steroid derivatives, or metabolic biproducts, and target specific domains within their cognate NRs. A nuclear receptor and its ligands orchestrate their effects on physiology largely by altering gene transcription in the nucleus, a process that drives pathology under a variety of conditions when improperly regulated. The prototypical nuclear receptor contains a ligand binding, DNA binding, and transactivating domain [3]. The DNA-binding domain contains two zinc fingers that bind to specific genomic sequences known as hormone response elements (HREs) [2]. Furthermore, ligand complexing with the nuclear receptor initiates structural changes within the ligand binding domain, exposing discrete sites to allow for the docking of coactivators, proteins that direct chromatin remodeling proximal to a target gene promoter [2,5,6]. As the precise cellular mechanisms vary between nuclear receptors, a more complete mechanism of nuclear receptor activity will be detailed hereafter (Sections 1.2.1 and 1.3.2).

1.1.2 Evolutionary Relationships within the Nuclear Receptor Superfamily

There is a significant amount of structural and functional variety between nuclear receptors. The Nuclear Receptors Nomenclature Committee developed a widely used standard

for naming nuclear receptors [4]. Each NR was categorized according to its evolutionary history, function, and general mechanism. There are currently 7 nuclear receptor subfamilies, and this dissertation will focus on two specific members (LXR and ER) found within two separate families (the first and third subfamilies), representing different general mechanisms and therefore different scientific considerations. Family one contains the thyroid hormone receptors (TRα/TRβ), retinoic acid receptors (RARα, RARβ, RARγ), peroxisome proliferator-activated receptors (PPARα, PPARβ, PPARγ), the liver-X receptors (LXRα and LXRβ), the vitamin D receptor (VDR), and the farnesoid-X receptor (FXR) among others. The third family contains the estrogen receptors (ERα, ERβ), glucocorticoid receptor (GR), mineralocorticoid receptor (MR), progesterone receptor (PR), and androgen receptor (AR). All the receptors mentioned within these two categories are known to bind a ligand [2-4]. Interestingly, there are nuclear receptors for which we have yet to identify a ligand, and those that could not possibly bind a ligand in the traditional sense as they lack an open ligand-binding pocket (such as Nurr1) [7]. These receptors are referred to as “orphan” nuclear receptors. There are also NRs that lack a DNA-binding domain (SHP for instance), and are not technically “transcription factors” but are still classified as nuclear receptors due to sequence homology with the nuclear receptor superfamily [1,4]. These receptors are very important for the activity of other NRs (such as LXR and FXR) and are not nuclear receptor pseudogenes.

1.1.3 RXR Dimerization Partner Receptors

Members of the large subfamily group I of nuclear receptors (TRα, TRβ, et al.) bind to DNA as heterodimers with the retinoid X receptors (RXRα, RXRβ, and RXRγ) [1,2]. The RXRs are activated by 9-cis retinoic acid ligands. Therefore, two activating ligands are required for optimal transactivation, conferring bimodal and combinatorial controls of nuclear receptor function, in contrast to group 3 nuclear receptors (ERα, GR), which generally bind to DNA as homodimers in response to a singular activating ligand [2,8]. The members of subfamily group I are thought to compete with one another for available ligand bound RXR, which raises questions

about the binding affinities for different isoforms of RXR and partner nuclear receptors, and how tissue specific expression of RXR isoforms and competition may reconfigure tissue specific responses to lipid ligands (PPAR), bile acid (FXR), and oxysterols (LXR) [4,9,10]. Regardless, RXR can form heterodimers to orient the nuclear receptor DNA binding domain to recognize palindromic, direct, or inverted repeat HREs [1,2]. The RXR and nuclear receptor dimerize along a hydrophobic repeat region lining the LBD, and is supported by a dimerization surface that forms upon DNA binding [2,11]. A set of RXR dimerization partners, the LXRs (LXRα/LXRβ), have been shown to regulate a wide variety of processes including reverse cholesterol transport, bile acid synthesis, lipid biosynthesis, as well as the proper secretion of pancreatic digestive juices into the intestine [12-15]. The LXRs are also involved in a variety of disease states. These receptors have been shown to regulate proliferation in a variety of cancers from different tissues upon activation with synthetic ligands. Critical processes in normal LXR function will be detailed, as well as its links to cancer cell proliferation, in the following sections.

1.2 Introduction: The LXRs in Health and Human Disease

1.2.1 Ligands and Transcriptional Regulatory Mechanisms of the LXRs

Two LXRs, LXRα (NR1H3) and LXRβ (NR1H2), were first identified as orphan NRs (or NRs lacking an activating ligand), and have been shown to heterodimerize with 9-cis retinoic acid receptors (RXRs) [16,17]. The LXRs have alternative tissue-specific expression profiles. LXRβ appears to be expressed ubiquitously, while LXRα expression is confined to the liver, intestine, lung, macrophages, kidney, and the adrenal glands [18]. Several endogenous agonists of LXR have recently been identified, such as 24-hydroxycholesterol, 20(S)-hydroxycholesterol, 22(R)-hydroxycholesterol, and 4β-hydroxycholesterol [19]. After their initial cloning and basic characterization, two synthetic agonists, GW 3965 and T 0901317, were developed [20,21]. These ligands bind to and activate both LXRα and LXRβ. An LXRβ-specific ligand has also been characterize, 5,6-24(S),25-diepoxycholesterol, as well as several LXR antagonists, such as

arachadonic acid and riccardin [22-25]. The identification of LXR ligands has facilitated the study of transcriptional regulatory mechanisms of the LXRs.

Generally, the NRs found in the NR superfamily group 1 (such as LXR), are thought to immediately translocate to the nucleus, where they bind to hormone response elements throughout the genome. In the classical mechanisms of NRs in subgroup 1, NRs recruit corepressors (i.e. SMRT) in the absence of ligand [1,2]. Corepressor complexes contain histone deacetaylases (HDACs), which remove transcription stimulatory signals on histone tails proximal to gene promoters. In the presence of a stimulating ligand, group 1 NRs recruit transcriptional coactivators instead of corepressors, leading to gene activation. However, chromatin immunoprecipitation experiments have shown that the LXRs bind to DNA in a ligand-dependent manner, much like estrogen receptor or other NR members of subgroup 3 [26]. Chromatin immunoprecipitation also demonstrate that LXR binds to many elements throughout the genome without an identifiable LXRE, suggesting that the canonical binding site for LXR is variable, or that LXR tethers to other transcription factors bound to DNA. LXRα and LXRβ bind preferentially to AGGTCAnnnnAGGTCA (LXRE), a direct repeat separated by four non-contacted bases. More detailed mechanisms of LXR and its regulated genes have led to a better understanding of the roles of LXR in physiology. Generally, the LXRs are known to regulate organism wide cholesterol levels by regulating the elimination of (1) cholesterol from the body, (2) the amount of cholesterol absorbed from ingested food, and (3) the amount of cholesterol synthesized in the liver [18]. Furthermore, due to their roles in regulating the amounts of serum triglycerides, lipoprotein particle assembly and regulation will be explored in Section 1.1.2 first. It is through these regulatory pathways that LXR modulation may result in better treatments for atherosclerosis, diabetes, or cancer.

1.2.2 The Role of LXR Ligands in Cholesterol Efflux

The LXRs promote bile acid synthesis of endocytosed LDL-associated cholesterols in the liver [18]. It was suggested that the LXRs regulated cholesterol metabolism in the liver due to (1)

the discovery of cholesterol metabolites as endogenous ligands of the LXRs, and (2) the identification of cholesterol ester accumulation in the liver of LXRα-/- mice [15,27]. These LXRα-/- mice were unable to convert effluxed cholesterol esters from peripheral tissues into bile acids. This accumulation results in murine hepatomegaly with extensive macrophage infiltration, which is suggestive of hepatitis [15]. Interestingly, LXRβ-/- mice do not accumulate cholesterol in the liver, but tend to have smaller adipocytes, and have impaired glucose-dependent insulin secretion [28,29]. Activation of the LXRs with synthetic ligands has been shown to both increase serum HDL levels, the antiatherosclerotic lipoprotein particle, and decrease net cholesterol levels in the peripheral tissues [30]. This effect depends on LXR-mediated induction of ABCA1 and ABCG1, leading to the export of fatty compounds to HDL throughout the body [31]. Furthermore, these basic mechanisms appear to occur in most, if not all, the tissues throughout the body.

These effects are due to diverse tissue specific effects of the LXRs, which have been well characterized in the liver, macrophages, and intestine. [32]. It was demonstrated that ABCA1 was induced in a mouse model of intestinal LXRα with constitutive activity (iVP16LXRα). ABCG5 and ABCG8 were also upregulated in these mice, which serves to limit the amount of dietary cholesterol absorbed. Constitutive LXRα mice were able to efflux cholesterol directly to HDL from the intestinal enterocytes. Expression of NPC1L1, which regulates the absorption of dietary sterols, was suppressed by constitutive LXRα activity. Overall, the absorption of dietary cholesterol was reduced in these mice, leading to increased hepatic cholesterol synthesis [33]. LXRα activation in the intestine is protective against atherosclerosis in LDLR-deficient mice, which suggests that the intestine is an important regulator in the pathogenic accumulation of fats in the arteries.

LXRs play an important role in regulating the use of these absorbed nutrients after uptake in the liver. Treatment of mice with LXR ligands has been shown to increase *de novo* lipogenesis in the liver [34,35]. This leads to increased increased lipid accumulation in the liver, as well as increased serum triglyceride levels (resulting in increased VLDL levels), which are the results of LXR-mediated upregulation of SREBF1, or sterol regulatory element-binding factor 1 [36].

SREBF1 is a basic helix-loop-helix transcription factor that regulates the production of fatty acids for export from the liver, and contains an LXRE in the gene promoter. SREBF1 upregulates fatty acid synthase, steroyl-CoA desaturase 1, and acyl-CoA carboxylase, key enzymes that mediate *de* novo lipogenesis [37]. Acyl-CoA carboxylase synthesizes malonyl-CoA from acetyl-CoA, byproducts of glycolysis. Fatty acid synthase catalyzes the formation of palmitate from malonyl-CoA and acetyl-CoA precursors. Steroyl-CoA desaturase introduces a double bond into the newly formed fatty acid, resulting in the generation of monounsaturated fats. Furthermore modifications result in the conversion of these fatty acids into phospholipids and triglycerides for direct use or export. This is thought to be the principle mechanism whereby the LXRs upregulate fatty acid synthesis.

LXR agonists have also been shown to reduce glucose tolerance in rodent models of diabetes [38,39]. First, PGC-1 levels in the liver are suppressed, which is an activator of gluconeogenic genes glucose-6-phosphatase, and phosphenolpyruvate carboxykinase (PEPCK). These downstream targets of PGC-1 are also repressed [38]. PEPCK generates a phosphoenolpyruvate molecule from oxaloacetate, and is the rate-limiting enzyme in gluconeogenesis [40]. Glucose-6-phosphotase hydrolyzes glucose-6-phosphate, yielding a phosphate group and a glucose molecule, and is the final step in gluconeogenesis [41]. Conversely, insulin sensitive glucose transporter GLUT4 is upregulated in adipocytes. Together, these data suggest that the LXRs activate transcription of genes involved in limited gluconeogenesis in the liver, and increase glucose uptake in peripheral cells through GLUT4 activation [38]. Enhanced LXRβ signaling in MIN6 pancreatic islet cells increased insulin secretion in the presence of glucose, as well as lipogenic genes fatty acid synthase and acetyl-CoA carboxylase [29,42]. This effect was attenuated in LXRβ-/- mice. LXRβ-/- mice tend to accumulate lipids in the islets themselves, and lose glucose dependent insulin secretion. To be clear, the LXRβ-/- mice are glucose intolerant, but not insulin resistant [29]. These data link LXRs and their ligands not only to lipid synthetic mechanisms in target cells, but also to glucose uptake and homeostasis in the endocrine pancreas.

Cholesterols that accumulate in the macrophages have been shown to function as endogenous ligands for LXR [18]. This results in the upregulation of *ABCA1* and *ABCG1* as a means to efflux cholesterol that has accumulated in the arteries to HDL particles. Both ABCA1 and ABCG1 contain *bona fide* LXREs, which mediate their induction upon ligand stimulation [43] [44]. A summary of the mechanisms in this section is depicted in Figure 1.1. In addition to their effects on cholesterol, blood glucose, and fatty acid synthetic gene networks, the LXRs are also known to modulate inflammatory signaling in macrophages and other tissues. A better understanding of these roles is of particular importance to atherosclerotic macrophages as well as general autoimmune diseases and immunogenic cancers. LXR ligands are able to repress inflammatory mediators, such as COX2, IL-6, MCP-1, and MMP9; in response to challenge with LPS (lipopolysaccharide) and TNF-α [45]. This effect is attenuated when both LXRα and LXRβ are knocked out, as there appears to be redundant compensation with these anti-inflammatory mechanisms when either is knocked out in isolation [45]. Interestingly, TLR3 and TLR4 activation result in the inhibition of LXRs through activated interferon-regulatory factor 3 [46]. LXR ligands have also been shown to decrease the severity of experimental autoimmune encepephalomyelitis [47]. In these treated mice, reduced expression of MHC II in the microglia, as well as reduced overall inflammation in the CNS. LXRαβ-/- mice experience more aggressive inflammatory responses when challenged with LPS treatment. Furthermore, it has been shown that LXR agonists improve symptoms in a model of contact dermatitis similar to steroid based anti-inflammatories such as dexamethasone [8,48]. Inflammatory mediators in macrophages do not appear to contain LXREs, which suggests independent tethering of LXR to other transcription factors. The LXRs likely transrepress selected NFκB response elements in a manner similar to other nuclear receptors, such as GR, ER, and VDR [46,49]. Taken together, these results demonstrate the centrality of the LXRs to body-wide cholesterol localization, bile acid synthesis, lipogenesis, response of the endocrine pancreas to blood glucose levels, and inflammatory signaling.

Due to the discovery that the LXRs target (an unintended) set of lipogenic genes, LXR agonists are thus far insufficient for widespread use in humans. However, because of their known roles in reverse cholesterol transport and inflammation, the LXRs still represent potential drug targets under the condition that more mechanism specific therapies are developed. There are examples of compounds that circumvent undesirable effects of particular NR ligands such as dissociated ligands that target specific transcriptional submechanisms of an NR, partial agonists, and modulators with mixed agonist/antagonist properties depending on the tissue. Ligands of these different varieties have been developed for estrogen receptor, glucocorticoid receptor, and androgen receptor. In the case of LXR, there is interest in developing a ligand that activates reverse cholesterol transport, but does not promote lipogenesis in the liver. Where LXRα-/- mice accumulate cholesterol in the liver hepatocytes, LXRβ-/- mice do not [15,28]. Therefore, there is interest in generating LXRβ-specific ligands, which may circumvent the lipogenic effects of LXRα. Despite a highly conserved ligand binding domain that is shared between LXRα and LXRβ, an LXRβ preferring ligand (179 nm for LXRα and 24 nm for LXRβ) has been developed (LXR 623) that promotes reverse cholesterol transport but does not induce lipogenesis in the liver [50]. These drugs proceeded to clinical trials but were halted after patients experienced central nervous system side effects, which is further evidence of extensive roles for LXR beyond the blood-brain barrier. Due to their widespread expression and their diverse effects on inflammatory and metabolic pathways, it makes targeting intended mechanisms more difficult. As these ligands have thus far have failed as treatments for atherosclerosis, diseases that have a much higher threshold for allowable adverse effects represent an opportunity for the further advancement of these ligands. For instance, these ligands are being explored as anti-cancer agents in a variety of different tissues. Many of these cancers have limited treatment options with a dim prognosis, such as cancer of the ovary, pancreas, and breast.

1.2.3 The Anti-proliferative Effects of Liver X Receptor Ligands

Nuclear receptors are currently the target of many compounds as they are highly druggable and can be modulated with compounds that target mechanisms of nuclear receptor function. Several nuclear receptors are targeted in the treatment of cancers of breast and prostate, such as ARs and ERs. ERs are targeted directly by small molecule anti-estrogens and selective estrogen receptor modulators (SERMs; tamoxifen; raloxifene) or indirectly by aromatase inhibitors (letrozole, exemestane, anastrozole) as a means to block endogenous estrogen production [51]. Because NRs have a hydrophobic pocket within the ligand-binding domain, they are able to bind a diverse set of compounds, natural or synthetic, that have unique effects on NR activity, physiology, and disease states [2]. This is in contrast to other non-NR transcription factors with inaccessible globular protein structures that significantly limit the development of small-molecule inhibitors.

Based on the well-characterized effects of ER on breast/ovarian cancer and AR on prostate cancer, it is likely that other NRs and their ligands regulate cancer progression in tissues throughout the body. Interestingly, it has been shown that epoxycholesterols accumulate in malignant prostate cancers [52,53]. Furthermore, SREBF1 and SREBF2, are upregulated in these prostate cancers. The presence of endogenous LXR ligands and activated SREBF1 suggested a potential role for the LXRs in prostate cancer development and progression. To test this hypothesis, mouse models of prostate cancer were treated with T0901317, which resulted in decreased tumor formation [54]. Overexpression of LXRα in these cells prior to injection resulted in increased sensitivity to the LXR ligand. Due to these observations in prostate cancer, the effects and mechanisms of the LXRs are being evaluated as potential therapeutic targets in diverse cancers.

After the initial characterization of the antiproliferative effects of LXR in prostate cancer, many studies have shown the effects of LXR ligands on cell cycle signaling pathways in diverse cancers. For instance, treatment of prostate cancer cells with LXR ligands leads to increased p27 protein levels, a cyclin-dependent kinase inhibitor [54]. SKP, an oncogene found in a large E3

ubiqutin ligase complex, was decreased in LNCaP cells after treatment with LXR ligands. Furthermore, SKP2 has been shown to target p27 for protesomal degradation, which is indicative of a potential antiproliferative mechanism. The effects on the protein levels in prostate cancer cell lines appear to be independent of a direct transcriptional mechanism, as the mRNA for these genes are unaffected by ligand treatment. It has further been shown that SKP2 transcript and protein levels are decreased in breast cancer cell lines, but p21 and p27 remain unaffected, suggesting alternative mechanisms of regulated by the LXRs between prostate and breast cancers [55]. LXR Ligand treatment and microarray analysis of breast cancer cell lines revealed an enrichment of repressed genes regulated by E2F2, which is also downregulated by LXR ligand treatment [56]. E2F family members are transcription factors integrally linked to cell cycle regulation. LXR agonists are also antiproliferative in breast cancer cell lines by disrupting both ER-dependent proliferation and cell cycle machinery [55,56]. Furthermore, LXR ligands have been shown to induce apoptosis in prostate, ovarian, breast cancers; and B cell leukemia [54,57,58]. These studies demonstrate a consistent effect of LXR ligands on cancer cell cycle and apoptosis pathways from a variety of cancers and tissues.

The effects of LXR ligands on cancer cell growth may be due to their well-documented effects on metabolism. Fatty compound transporter ABCA1 has been shown to be downregulated in malignant prostate cancer cells [59]. Knockdown of this transporter has been shown to increase prostate cancer cell proliferation. Interestingly, ABCA1 is an induced target of the LXRs, and may provide a mechanistic link between LXR ligands and the observed effects on cell proliferation. ABCG1, another cholesterol transporter and induced target of LXR, has been shown to increase AKT1 signaling, resulting in apoptosis in prostate cancer cell lines [60]. Activated targets of LXR, ABCA1 and ABCG1, are known to efflux excess cholesterol to HDL particles. As part of this mechanism, LXR ligands have been shown to downregulate LDLR expression, which accepts lipoprotein particles consisting primarily of cholesterol used in downstream mechanisms [61]. Both these mechanisms exist to limit cholesterol uptake. Suppression of LDLR expression through LXR treatment resulted in apoptosis in glioblastoma cells, and represents another

potential mechanism for the antiproliferative effects of the LXRs. Apolipoprotein E (APOE), which is found in chylomicrons and IDLs, is necessary for the transport of fats through the blood. APOE is transcriptionally activated by LXRs and RXRs. Upregulated APOE is associated with suppressed metastasis in melanoma cancer cells [62]. Therefore, Studies of LXR ligands in colon, breast, prostate, lung, and skin cancer cells indicate a potential role for these ligands and LXRs in cancer cell proliferation [63].

Knockout animals provide a starting point to ascertain what roles the LXRs play in carcinogenesis. Female mice lacking LXRβ spontaneously undergo a process of gallbladder transformation suggesting a specific role of this receptor in regulating carcinogenesis [64]. There are also roles for LXRβ in the pancreas, as LXRβ knockout mice have reduced lipase, amylase, and protease secretion into the upper intestine, resulting in pancreatic insufficiency and malabsorption. This abnormality is also accompanied by large infiltration of immune cells in the pancreatic ducts, possibly because of LXR's known roles in suppressing inflammatory responses. Examination of the tissue sections revealed pancreatic ducts reminiscent of chronic pancreatitis in humans, a risk factor for pancreatic cancer. In these particular mice, there was no difference in cell proliferation between wild-type and knockout mice, but there was increased apoptosis in the pancreatic ducts. An antiproliferative effect of LXR ligands we recently demonstrated in pancreas. A low dose of GW 3965 was potentiated by treatment with 9-cis-retinoic acid in pancreatic islet cells [65]. These studies, however, were carried out in non-transformed cells. The effect of LXRs on malignancy in the pancreas of exocrine duct origin has not yet been explored, and represents a unique opportunity to explore the roles of nuclear receptor ligands in pancreatic ductal adenocarcinoma.

1.2.4 Pancreatic Ductal Adenocarcinoma and Currently Approved Therapies

The pancreas is a key organ involved in digestion and blood sugar regulation, and is subject to a wide variety of pathological conditions, which are known to dramatically reduce the quality and length of life. Pancreatic cancer is an umbrella term used to describe malignancies derived from cells in the pancreas, and is composed of several different subtypes with varying

outcomes and prognoses[66]. Pancreatic ductal adenocarcinoma (PDAC) is the most common type of pancreatic cancer, and originates from the ductal cells that carry digestive enzymes and basic solutions to the intestines. Pancreatic neuroendocrine tumors (i.e. insulinomas) arise from cells in the Islet of Langerhans, and are less common and more treatable pancreatic cancers [67]. Malignant tumors derived from pancreatic acinar cells are relatively rare when compared to PDAC (~5%) as well. Cancers of the pancreatic ductal cells (PDAC) are highly resistant to available therapeutics, with a combined survival rate of 5% after five years regardless of the initial stage of the pancreatic cancer at the time of diagnosis [68]. PDAC is an aggressive and resistant cancer, often failing to respond to the standard-of-care chemotherapeutic gemcitabine, a cytidine nucleoside analog that blocks DNA replication. Approximately 20% of the diagnosed cases of PDAC are resectable with the intention of curing the cancer [69]. As many of these cancers arise in the “head” of the pancreas, a patient may undergo an operation to remove the duodenum and the gallbladder in addition to the head of the pancreas due to a shared blood supply [70,71]. The tail of the pancreas and the stomach are then reattached to the jejunum. Such a dramatic remodeling of the digestive system is more or less tolerated depending on the amount of pancreas removed. Patients with extensive pancreas removal may not produce enough digestive enzymes to properly break down food particles, and may also experience mild diabetes. These surgical procedures are risky and very complicated due to individual variations in the configuration of the main pancreatic ducts.

Malignant PDAC tumors are thought to be derived from pancreatic intraepithelial neoplasias (PanIN), which are positively correlated with age and chronic pancreatitis [72]. PanINs are found mostly in the head of the pancreas [73]. In post-mortem autopsies, PanINs were remarkably common, and are divided into different stages, representing different levels of dysplasia [74]. PanIN-1 lesions are histologically columnar that have maintained polarity. Cells that have lost cell polarity and contain hyperpigmented nuclei of variable size are referred to as PanIN-2. The most complex lesions are PanIN-3 grade, which contain cell nuclei that are enlarged and lack orientation [74]. Structurally, these cells form papillae, with epithelial buds

protruding into the ductal lumen. Mutations leading to higher-grade neoplasias are thought to be acquired sequentially, albeit there are no exact or preordained routes leading to high grade PanINs [72]. For instance, KRAS2 hyperactivation leads to increased cell division and extensive telomere shortening, and is thought to be an early event in dysplasia leading to PanIN-1. p16/CDKN2A is then lost, leading to the development of PanIN-2 lesions. Bi-allelic loss of P53 tumor suppressive activity is associated with the development of PanIN-3. Many of these genes have known roles as tumor suppressors or oncogenes, thereby supporting the notion that PanINs are early pancreatic cancers.

After the resection and remodeling of the remaining pancreas, patients undergo rounds of chemotherapy after sufficient recovery time (1-2 months). A majority of patients (~80%) are not eligible for surgical resection due to the advanced nature of the disease at the average time of diagnosis, but are offered chemotherapy to extend lifespan and to improve the quality of life [69]. Gemcitabine, the standard chemotherapeutic for pancreatic cancer, was found to be more effective that 5-fluoruracil, another nucleotide analog that introduces double stranded breaks into the DNA of rapidly dividing cells [75]. Gemcitabine improves median survival just over one month when compared to 5-fluorouracil [76], and was the first chemotherapeutic agent ever approved by the FDA based on parameters other than the survival endpoint. Recent advances in PDAC treatment paired gemcitabine with EGFR inhibitors, such as erlotinib or cetuximab [77,78]. Erlotinib marginally improves the median survival by only two weeks. EGFR inhibitors are typically used in patients with overexpressed or mutated EGFR in a variety of different cancers. The success of EGFR therapy is dependent upon an unmutated/wild-type KRAS due to constitutive growth factor signaling independent of and downstream of EGFR with mutated KRAS. In general, EGFR inhibitors have been shown to be subject to the rapid development of resistance approximately 10 months after treatment initiation, and by themselves, do not represent a viable strategy for the treatment of cancers in the long term [79]. Therefore, alternative strategies other than those currently approved are needed to improve the survival and quality of life of pancreatic cancer patients. Data that demonstrate the antiproliferative effects of

the LXRs in other tissues represent potential treatment avenues in the treatment of pancreatic cancers. The associated study is presented in Chapter 2.

1.3 Introduction: Mechanisms of ERs in Breast Cancers[1]

1.3.1 Estrogens and Estrogen Receptors: The Initial Characterization of Estrogen

The female steroid hormone estrogen has pleiotropic effects on human development, physiology, and a number of endocrine-related conditions and diseases [80-82]. Initially, estrogen was thought to be involved in cellular redox reactions through interactions with coenzymes[83]. A competing hypothesis suggested that cells in target tissues may harbor receptor molecules whose presence would dictate tissue-specific responses [84]. Jensen and Jacobson developed a method to tritiate estradiol, the predominant form of estrogen, and showed that radiolabeled hormones were functional, accumulated in estrogen-responsive reproductive tissues, and were not chemically altered [85]. Studies by Gorski et al. and also Jensen et al. went on to demonstrate that estrogen was bound to proteins in the cytoplasm that subsequently localized to the nucleus of target cells and activated the synthesis of specific transcripts [86-89]. Jensen termed the estrogen-binding protein estrophilin, whereas Gorski referred to the protein as the estrogen receptor (ER) [90,91]. Molecular characterizations of ER became possible when the ER gene was cloned by the Chambon group [92]. Mutagenesis studies showed that the receptor consists of a DNA-binding domain containing zinc finger motifs and a ligand-binding domain, key structural elements of a ligand-dependent transcription factor [93]. Other related receptors, including the glucocorticoid receptor, the thyroid hormone receptor, and the progesterone receptor, were also cloned and characterized around the same time [94-97]. A second closely related ER with similar affinity for estradiol but distinct tissue specificity and affinity for other estrogenic compounds was discovered by Gustafsson and Kuiper and was subsequently named ERβ, and the original ER was renamed ERα [98]. The roles of ERα and ERβ in mediating estrogen response in normal

[1] Contents in this chapter have been previously published as “Estrogen receptor alpha: Molecular

physiology and in diseases have been subjected to intense investigation. An orphan G protein-coupled receptor (GPR30) was also reported to function as an ER, but these observations have been disputed by more recent hormone binding and genetic studies [99].

1.3.2 Transcriptional Regulatory Mechanisms of ER

Upon activation by ligand, ERα dimerizes and binds directly to *cis*-regulatory regions of target genes via conserved estrogen response elements (EREs; consensus 5′-GGTCAnnnTGACC-3′) [100]. ER can also be tethered to other transcription factors such as AP-1, Sp1, and NFκB to indirectly influence gene expression [101-103] (Figure 4.1B-C). Structural changes induced by ligand binding to ERα facilitate the formation of nuclear receptor co-regulator binding surfaces which then complex SWI/SNF ATP-dependent nucleosome remodeling enzymes that enable necessary changes to histone spatial position and co-activators which include histone modifying enzymes such as histone acetyl-transferases (HATs: P300/CBP, P/CAF, SRC-1, and p/CIP/AIB1), histone methyl-transferases (HMTs: CARM1 and PRMT1), and histone ubiquitin ligases (RPF1 and E6-AP) [5,104]. Co-repressors, such as NCoR, NRIP1, and SMRT recruit histone deacetylases (HDACs) and have been shown to bind to ERα in the presence of antagonists or at specific *cis*-regulatory regions of repressed target genes following hormone activation [105-107]. These interactions and mechanisms are summarized in Figure 1.2.

Once the proper histone modifications are made at the target gene promoter orchestrated by ERα-bound coactivators, TFIID/TBP is stabilized at the promoter by TFIIA. TFIIB then binds and positions RNA polymerase II at the correct initiation site [108]. Signaling of TFIIH by the TRAP/mediator complex stimulates phosphorylation of the C-terminal domain of the polymerase leading to transcription initiation and elongation. SWI/SNF is typically maintained at the promoter as the cells utilize this complex to modify histone spatial configuration in preservation of active transcription in the presence of ERα, but also to discontinue that configuration once estrogen response is uncoupled [109]. After co-activator assembly, members of the ubiquitin proteasome pathway regulate degradation of portions of the preinitation complex so that elongation is possible

[6]. Treatment of estrogen-responsive cells with proteasome inhibitors interrupts the cyclic association of co-regulator proteins with the ERα complex, which eventually results in the loss of phosphorylated polymerase II. ERα itself is subjected to a variety of post-translational modifications which result in changes in receptor stability and activity in response to specific ligands and signaling events in the cell [110].

1.3.3 Non-genomic Actions of Estrogen Receptor

ERα is predominantly studied as a ligand-dependent transcription factor, but early work demonstrated that estrogen has rapid effects on cell signaling [111-114]. These effects suggested the existence of signaling pathways not mediated by transcriptional regulatory mechanisms. Estrogen rapidly signals ERα/G protein complexes through secondary messengers calcium and cAMP, activated PI3K, and activated RAS [113]. Some ERα appear to localize to the cytoplasm and interact with growth factor receptors (GFRs) such as EGFR and IGF-1R. Activation of these receptors through ERα is thought to stimulate GFR effector kinases and their downstream targets such as PI3K and ERK. Activation of PI3K by GFR/ERα signaling results in the inhibition of GSK-3β, which is known to phosphorylate ERα S118 and inhibit its activity. This allows for amplified ERα transcriptional activity in the nucleus [112]. It is through these GFR effector signals that estrogen is able to upregulate cyclin D1 expression, thereby promoting G1/S transition through the cell cycle.

ERα is believed to be tethered to the cytoplasmic membrane through one or a combination of different mechanisms, such as association with lipid-raft proteins like caveolin-1 or by direct interaction with GFR complexes [114,115]. Interaction of ER with caveolin-1 and the cell membrane requires palmitoylation of ERα [116]. Interestingly, this modification is required for signaling ERK and PI3K pathways. Recent experiments have attempted to characterize a separate membrane bound receptor for estrogen, as almost all ER is found in the nucleus. In fact, membrane bound ER was thought to be GPR30. However, knockdown experiments failed to prevent ERK activation after estrogen stimulation, raising questions about the significance of this

finding [117]. Several lines of evidence suggest that membrane-bound ER appears to be the same ERα and ERβ found in the nucleus [113]. The non-genomic mechanisms of ERα action are depicted in Figure 1.2.

1.3.4 Novel Transcription Factor Binding Partners

The genome-scale ERα binding site mapping studies yielded a large amount of positional and sequence data and provided rich datasets for computational modeling and analysis of sequence motifs associated with ERα binding. For example, motif analysis confirmed the previously defined ERE consensus sequence motif [118,119]. Furthermore, other sequence motifs are enriched in the proximity of ERα binding sites and suggest potential physical and functional interactions between ERα and other transcription factors. The first chromosome-wide mapping studies revealed an enrichment of binding site motifs for Forkhead factors [120]. This enrichment was subsequently confirmed by genome-wide mapping studies [118]. Forkhead family member FOXA1 was previously identified as an ERα interacting protein and was shown by Carroll et al. to be the Forkhead factor which localize to the proximity of ERα binding sites and serve as a pioneering factor which potentiate ERα binding and transcriptional activity. FOXA1 appears to play key roles in hormone-dependent tumor growth and response to endocrine therapy in breast cancer and has similar roles in prostate cancer through its interactions with the androgen receptor [121,122]. In another study of binding site motifs associated with ERα binding sites, Kong et al. identified the transcription factor GATA3 as a component of an enhanceosome which includes both ERα and FOXA1, and this complex is involved in optimization of transcriptional responses to estrogen treatment [123]. Interactions between GATA3 and ERα also involve a cross-regulatory mechanism by which each of these factors regulates the expression of the other through direct binding of cis-regulatory regions of their partners [124]. Related to these molecular interactions, GATA3 has established roles in mammary gland development and its expression is highly correlated with ER-status in breast tumor tissues [125,126]. In a follow-up study to the ERα-promoter interactome mapping paper, Tan et al. identified AP-2 binding site

motifs in ERα binding sites which are involved in long distance looping structures and showed that AP-2 γ is a collaborative factor in interactions involving ERα, FOXA1, and promoter regions of target genes [127]. AP-2 γ functions in hormone response in breast cancer cells and its expression levels are elevated in tumors with poor clinical outcomes [128,129].

Adding to the rapidly accumulating datasets and insights, Stender et al. carried out a genome-wide ERα binding site study using an ERα construct with mutations in the DNA-binding domain specifically designed to detect tethering mechanisms and factors [130]. They showed that in addition to the binding site motif of the AP-1 complexes, known to tether ERα to DNA, there is an enrichment of binding site sequence motifs for runt-related transcription factors. They also provided evidence that RUNX1, specifically, binds ERα and regulates the expression of a subset of estrogen-responsive target genes. RUNX1 has been well studied for its roles in hematopoiesis and regulation hematopoietic stem cell differentiation. RUNX1 is implicated in leukemogenesis and is frequently mutated in malignant myeloid cells, and there is increasing evidence for its role in breast cancer [131,132]. Interestingly, a genetic study in mice examining strain-specific uterotropic responses to estrogen treatment identified quantitative trait loci which included the locus for the Runx1 gene and showed a correlation between Runx1 transcript and protein expression levels and response to hormone treatment [133]. The transcriptional mechanisms of ERα action are depicted in Figure 1.2B-C.

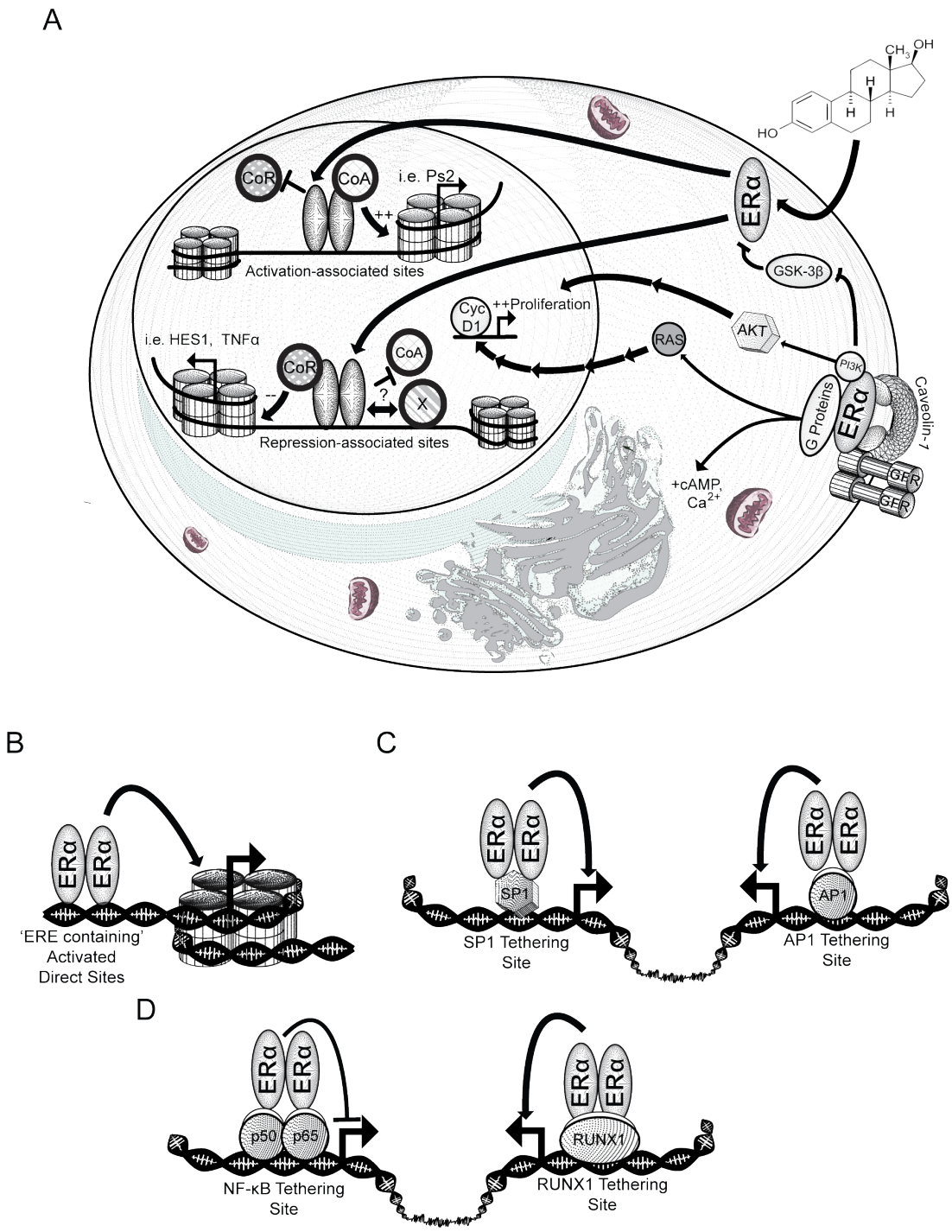


Figure 1.1 Mechanisms of action of ERα in the nucleus and cytoplasm of a target cell.
(A) Genomic and non-genomic actions of estrogen receptor. (B) Direct DNA binding activity of estrogen receptor to EREs. (C), (D) Tethering of ER to alternative transcription factors throughout the genome.

1.3.5 Breast Development

The breast is composed of glandular and parenchymal tissue (stromal cells). The non-parenchymal breast is composed of ducts and lobules, which produce milk that is channeled into the ducts. Myoepithelial cells surround the ductal cells, and contract during lactation to promote movement of secreted milk throughout the duct system. The first observable mammary structures are termed the milk lines, which are specified by Wnt10b [134]. Followed by this, placodes are specified, and are programmed to invaginate the mesenchymal tissue underneath them by FGF10 and BMP4 [135]. Parathyroid hormone-related protein (PTHrP), which is secreted from the mammary bud, regulates BMPR1A in the mammary mesenchymal cells, thereby sensitizing them to BMP4 signalling [136]. Activated BMP4 signaling in the mammary mesenchymal cells regulate the invasion of the epithelial and mesenchymal mammary cells into the fat pad precursors. At this point, this structure arrests invasion until puberty, which is primed to further elongate into the fat pad dependent upon estrogen signaling. Gata3 has been shown to be important for terminal end bud formation, which in combination with FOXA1, potentiate breast-specific targets of ERα [125]. Terminal end buds do not form and do not invade when ERα or Gata3 are attenuated. During puberty, EGF, estrogen, and CSF1 mediate the elongation and invasion of tubular ducts past the lymph nodes until the end of the mammary fat pad [137]. Terminal end buds (TEBs) are found at the ends of these ductal structures. During early pregnancy the terminal end buds no longer proliferate, but are programmed to develop side branching alveoli. This transformation is regulated by GATA3, prolactin, and progesterone receptor (PR). Side branching is attenuated in PR knockout models. Upon childbirth, the luminal cells are able to generate milk. Lactation is promoted by GATA3, prolactin, and STAT5A/B. Expanded breast tissue is involuted upon weaning, which results in the ordered apoptosis of breast tissue back to the pubertal size.

1.3.6 Breast Cancer Subtypes and Treatments

The invasive nature of the breast placode in early specification suggests that there are developmental pathways that are often reactivated constitutively by cancers. Advances in genome-wide sequencing in the new millennia has allowed for the characterization of individual tumors based on gene expression. Four major types of breast cancer have been identified based on these gene expression profiles: Estrogen receptor α, progesterone receptor, HER2 expression levels, and Ki67 expression levels. First, ER expression confers a better prognosis and an additional panel of drugs to treat them [138,139]. ER dependent cancers are treated with selective estrogen receptor modulators (SERMs), which are designed to attenuate estrogen dependent cell proliferation. ER+ breast cancers are also treated with aromatase inhibitors, compounds that prevent *de novo* synthesis of estrogens within the breast cancer itself [51]. Second, progesterone receptor, which regulates branching morphogenesis in lactation, is predictive of a normally functioning ERα and response to SERM compounds [140]. Thirdly, HER2/ERBB2 is a cell surface growth factor receptor that is overexpressed in 30% of breast cancers [141]. Hyperactive HER2 signaling results in increased MAPK signaling, markers that are known to decrease the antiproliferative effects of SERMs. These receptors are attenuated by monoclonal antibodies, such as trastuzamab and pertuzumbab, which are approved for the treatment of HER+ breast cancers. Lastly, Ki67 staining is a well-established marker for cell proliferation, and its expression levels are negatively correlated with survival and response to therapy [142]. These markers allow for the tailoring of treatments to attain the best possible outcomes with currently available therapeutics.

Breast cancer cells express these four markers in different arrangements or combinations. This configuration largely determines their response to different therapies, and may also denote the cellular origin of the malignancy. Cancers that are ER+/PR+/HER2-/Ki67^{low} are categorized as luminal A breast cancers. This molecular subtype represents 40% of the diagnosed cases, has the best prognosis of the explored cancers, and resembles cells that line

the lumen of the mammary duct [138,139]. These cancers are treated with endocrine therapy and have relatively low recurrence. The luminal B subtype is ER+/PR+/HER2+/Ki67^{low} or ER+/PR+/HER2-/ Ki67^{high} and represents 20% of the diagnosed breast cancer cases [138,139]. These cancers are also derived from the cells lining the mammary ducts, but proliferate more rapidly, and therefore are characterized by less favorable outcomes than luminal A breast cancers. Triple negative breast cancers are ER-/PR-/HER2- and encompass 20% of diagnosed breast cancers. Triple negative cancers are derived from cells that surround the mammary ducts, and usually bare p53 mutations, which are predictive of poor response to therapy [138,139]. These cancers are limited to chemotherapeutics due to the lack of the targetable markers mentioned earlier. Triple negative cancers are associated with a poor prognosis when compared to luminal A and B. Lastly, the HER2 type is ER-/PR-/HER2+ and is found in the remaining 10-15% of diagnosed women [138,139]. These cancers also have a poorer prognosis than luminal A and luminal B, but can be targeted with trastuzamab, which leads to decreased cell proliferation. These different breast cancers represent major subtypes, and are each predisposed by different genetic, environmental, and behavioral factors.

Risk factors for the development of breast cancer are first a foremost, older age and female sex. Cancer is thought of as a disease of aging, as mutations accumulate in cells throughout the body, the chances of a cell acquiring the correct mutations and the precise environmental conditions increase. Female sex predisposes women to cancer due in large part to the presence of estrogen, which regulates the development of the breast duct [143]. Excessive levels of estrogen, early puberty, or hormone replacement therapy for women in menopause, are known to increase the risk of breast cancer [144]. Secondary to these are dietary, genetic, and behavioral factors. Mutations in several genes have been shown to dramatically increase the risk of breast cancer. The most potent, however, is BRCA1, a tumor suppressor involved in DNA damage repair. Mutations in this gene result in a hereditary breast-ovarian cancer syndrome [145]. These women are at incredible risk of several malignancies, as high as 80% will develop breast cancer by the age of 90 [146]. BRCA1 mutations also predispose women to an increased

risk of ovarian cancer (~25%). Modifiable behavioral factors such as alcohol consumption have been shown to increase the risk of breast cancer. Epidemiological studies have strongly linked alcohol consumption to increased breast cancer risk [147-150]. These epidemiological studies, therefore, will attempt to more clearly link alcohol with measurable phenotypes in cell-based assays as a means to clearly identify mechanisms of alcohol action in breast cancer cells. These studies are presented in Chapter 3.

2. Antiproliferative Effects and Mechanisms of Liver X Receptor Ligands in Pancreatic Ductal Adenocarcinoma Cells[2]

2.1 Introduction

Pancreatic ductal adenocarcinoma (PDAC) is among the most deadly cancers, with a combined (all four stages) survival rate of 5% after five years [68]. Localized neoplasms represent about 20% of diagnosed cases and are resected using the Whipple procedure [69]. PDAC is often asymptomatic until the disease is late in its progression and tends to be poorly vascularized and resistant to the standard-of-care chemotherapeutic gemcitabine, a cytidine nucleoside analog that blocks DNA replication [75]. Gemcitabine improves median survival just over one month when compared to 5-fluorouracil [76]. Recent advances in PDAC treatment paired gemcitabine with EGFR inhibitors, such as erlotinib or cetuximab, and this combination improved median survival by less than two weeks [77,78]. Alternative strategies are clearly needed to improve survival and quality of life for PDAC patients.

Members of the nuclear receptor (NR) superfamily of ligand-dependent transcription factors carry out vital cellular functions and are highly druggable targets [151]. NRs are modulated by steroidal and non-steroidal compounds in maintenance of normal metabolism, development, and immune responses [2,152]. Because NRs have ligand-binding domains with highly specific binding pockets, they can be targeted by a plethora of synthetic compounds in the treatment of autoimmunity, diabetes, and hormone-dependent malignancies of the breast and prostate [2,152]. For example, estrogen receptor plays a key role in breast cancer and is targeted by selective estrogen receptor modulators (SERMS) in the prevention and treatment of hormone-dependent breast cancers [153]. The androgen receptor is similarly targeted in the treatment of prostate cancers.

[2] This chapter of the thesis has been previously published as the "Antiproliferative Effects and Mechanisms of Liver X Receptor Ligands in Pancreatic Ductal Adenocarcinoma Cells" in *PloS One*, *9*(9), e106289.

Liver X receptors (LXRs) are members of the nuclear receptor superfamily and have been studied extensively for their roles in regulating cholesterol, glucose, fatty acid metabolism, and inflammatory related pathways [2]. Two isoforms have been described, LXRα and LXRβ, that despite common characteristics (high homology in sequence, heterodimerization with 9-cis retinoic acid receptors, and a similar ligand profile) have distinct and specific functions [154]. LXRs are activated by a variety of endogenous ligands in normal homeostasis (27-hydroxycholesterol, 20(S)-hydroxycholesterol), or by synthetic ligands such as GW3965 or T0901317 that were developed for the treatment of atherosclerosis. Recent studies in rodents have shown that LXRβ is strongly expressed in pancreatic ductal epithelial cells and LXRβ-/- mice develop a severe pancreatic exocrine insufficiency [12]. However, it is not know whether LXRβ or its ligand may affect normal exocrine pancreatic function or the development of malignancies in humans. Studies of LXR ligands in colon, breast, prostate, lung, and skin cancer cells indicate a potential role for these ligands and LXRs in cancer cell proliferation [63]. Treatment of LNCaP prostatic cells with LXR agonists suppressed their growth in xenograft models [54]. LXR agonists are also antiproliferative in breast cancer cell lines by disrupting both estrogen-dependent proliferation and cell cycle machinery [55,56]. In addition, female mice lacking LXRβ spontaneously undergo a process of gallbladder carcinogenesis suggesting a specific role of this receptor in regulating cell proliferation [64]. Interestingly, an antiproliferative effect of LXR ligands is potentiated by treatment with 9-cis-retinoic acid in pancreatic islet cells[65]. Based on these observations, we hypothesized that LXR ligands may block cancer cell growth in PDAC. In this study, we examined the effects of LXR agonists on PDAC cells and identified potential mechanisms of action.

2.2 Materials and Methods

2.2.1 Ethical Statement

De-identified human samples utilized in the study were obtained from the Texas Cancer Research Biobank (http://txcrb.org/index.html) that collected the samples following patient

consent and collection protocol (H-29198) approved by the Baylor College of Medicine Institutional Review Board. The use of the tissues by the authors was exempt from institutional review as confirmed by the University of Houston Institutional Review Board.

2.2.2 Immunohistochemistry

Representative sections (n=8) of pancreatic adenocarcinoma were obtained from Texas Cancer Research Biobank. 4 males and 4 females were studied (age 40-69). Sections were dewaxed in xylene and rehydrated through graded ethanol. After antigen retrieval with PT module (Thermo Scientific) for 17 minutes at 97 °C, sections were incubated in 3% H_2O_2 in 50% methanol for 30 min at room temperature to quench endogenous peroxidase. To block nonspecific binding, sections were incubated in PBS containing 1% BSA and 0.1% Nonidet P-40 for 1 h at room temperature. Primary antibody reactions were incubated at 4 °C overnight. Goat anti-LXR β and anti-LXR α antibodies were developed as previously described [12,155] and used at 1:50 dilution in 1% BSA and 0.1% Nonidet P-40. Negative controls were incubated with PBS containing 1% BSA and 0.1% Nonidet P-40 without primary antibody. After washing, sections were incubated with goat-probe (Biocare Medical, GHP516) for 15 minutes, then washed in PBS and incubated with goat-on-rodent-HRP polymer (Biocare Medical, GHP516) for 15 minutes. After washing in PBS, sections were developed with 3,3'-diaminobenzidine tetrahydrochloride substrate (DAKO) and then counterstained with Mayer´s hematoxylin. Sections were dehydrated through a graded ethanol series and xylene and finally mounted.

2.2.3 Cell Lines and Tissue Culture

Three human pancreatic cancer cell lines were selected for these studies, BxPC-3, MIA-PaCa-2, PANC-1, (American Type Culture Collection, Rockville, MD, USA). MIA-PaCa-2 and PANC-1 were grown in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) containing high Glucose with HEPES and supplemented with 10% fetal bovine serum. BxPC3

cells were cultured in DMEM F-12 (Invitrogen), containing HEPES and Glutamine and supplemented with 10% FBS (Hyclone, Logan, UT, USA).

2.2.4 Cell Treatments, Gene Knockdowns, and Cell Proliferation Assays

Cells were treated with GW3965 (Tocris Bioscience, Bristol, UK), T0901317 (Tocris Bioscience, Bristol, UK), gemcitabine (Sigma-Aldrich, St. Louis, MO, USA) at indicated concentrations or ethanol as a vehicle. Cell proliferation was measured by MTS metabolic rate assays using CellTiter96® AQueous One Solution (Promega, Madison, WI, USA) following manufacturer's protocol or standard trypan blue exclusion assays using the Countess automated cell counter (Invitrogen) or hemocytometer. Experiments were performed in triplicate. LXR knockdown experiments were performed by transfecting PDAC cells with pooled targeting siRNA against LXRα and LXRβ following manufacturer's (Thermo Scientific Dharmacon, Lafayette, CO, USA) protocol. Transfections with scrambled siRNA were included as negative controls.

2.2.5 Cell Cycle Analysis and BrdU Incorporation Assays

Cells were treated with 10 μM GW3965 for 72 hours and then pulsed with 10 μM BrdU for 1 hour. Treated cells were then trypsinized and fixed in 70% ethanol and stored at -20° C for 24 hours. DNA was denatured in 2 M HCl/0.5% Triton-X and then neutralized in 100 mM sodium borate. FITC-conjugated anti-BrdU antibody was then added to bind incorporated BrdU. Fixed cells were incubated at 37°C for 30 minutes with 50 μg/ml of propidium iodide and 10 μg/ml RNase A. FACS Aria 111 Cell Sorter (BD Biosciences) utilized for data collection, and the data were analyzed using FlowJo software program.

2.2.6 Clonogenic Assay

Cells were seeded in 100mm plates and treated with LXR ligand for one week (MIA-PaCa-2) or two weeks (BxPC-3 and Panc-1). At the end of treatment period, cells were washed

with PBS and fixed in 4% formaldehyde and washed again with PBS. Colonies were then stained with crystal violet (Sigma-Aldrich), scanned, and quantified using the Clono-Counter software [156].

2.2.7 Microarray and Data Analysis

Total RNA from each cell-line was isolated using RNeasy columns (Qiagen). The Illumina TotalPrep-96 RNA Amplification kit was used to convert 250 ng of RNA to cRNA (Ambion, Carlsbad, CA, USA). Then, cRNA was hybridized to the Illumina Whole-Genome Gene Expression Direct Hybridization microarray (Illumina, San Diego, CA, USA). Probes that detect multiple genes were eliminated. The R software packages *lumi* and *limma* were used to calculate differentially expressed genes in treated cells. Intensity values were normalized and log-2 transformed. The Benjamini-Hochberg correction was used to correct for potential false discovery. A 1.1 fold change cutoff was then used to generate a list of responsive genes for data mining. Bioinformatic analyses of enriched gene sets were made in Pathway Studio (Ariadne Genomics, Rockville, MD). Fisher's exact test was applied to determined significantly enriched pathways. Transcription factor (TF) target enrichment, gene ontology (GO) categories, and Ariadne Pathway Categories used were provided within the software. The microarray data have been deposited with the Gene Expression Omnibus repository and will be available for public access following publication (accession number GSE51656).

2.2.8 Quantitative PCR

RNA was extracted using a Qiagen RNeasy kit then reverse transcribed using SuperScript III reverse transcriptase system (Invitrogen). Quantitative PCR was then performed using Fast SYBR Green Master Mix (Applied Biosystems, Carlsbad, CA, USA) on a 7500 fast real-time PCR system (Applied Biosystems). Primers for these genes were designed using Primer BLAST. These are listed in Table 2.1. Fold changes were

calculated using the ΔΔCt method normalized to 36B4, a housekeeping gene (*36B4* forward, 5'-GTGTTCGACAATGGCAGCAT-3'; *36B4* reverse, 5'-GACACCCTCCAGGAAGCGA-3').

2.2.9 Western Blot Analysis

Cells were serum starved 24 hours prior to treatment and restoration to normal medium. Ligand-treated cells were lysed in RIPA lysis buffer. Protein concentrations were measured using Qubit Protein Assay Kit (Invitrogen). 50 µg of protein was loaded into standard 10% polyacrylamide gels. After protein separation, SDS-PAGE gels were transferred to PVDF membranes (Millipore, Billerican, MA, USA). Membranes were then blocked in 10% nonfat milk dissolved in TBST than probed with antibodies directed against LXRα (proprietary, C. Gabbi), LXRβ (GeneTex Cat no. 89661), Skp2 (Santa Cruz sc-7164), EGFR (Santa Cruz sc-03), phospho-EGFr (Tyr1173) (Invitrogen 18-2465), ERK1/2 (Cell Signaling 9102), phospho-ERK1/2 (Thr202/Try204) (Cell Signaling 4377),or β-actin (Sigma-Aldrich A2228) in 1% milk overnight. Membranes were then washed of unbound antibody and reprobed with secondary antibodies conjugated to horseradish peroxidase (HRP) for at least 1 hr. HRP bound antibodies were then exposed to ECL reagent (Thermo Fisher Scientific, Rockford, IL, USA), which allows for their detection by film. Fold change quantification was determined by densitometric analysis available in ImageJ software.

Table 2.1 Primers used in this chapter		
Gene	**Forward**	**Reverse**
RFC2	5'-CAGCAAGCCTTGAGGAGAA-3'	5'-GCACAGCGGGACTGAATG-3'
CLSPN	5'-ACGGTTTACTCTGGATAGA-3'	5'-TCACCTCTGTTGGTTTCA-3'
PKIA	5'-CTGGACAAATAGCAGACAATG-3'	5'-CTGGCACAACCACACAAA-3'
MCM3	5'-GTCTGTGTGGAGGGCATTG-3'	5'-CGTCGCTCTATGGTCTTCTT-3'
JAG2	5'-TCAGAGGCAAGGTCAGCATTT-3'	5'-CAAGCAGTGAGGGGCAAAAC-3'
STAT3	5'-CAGCAGGAGGGCAGTTTGA-3'	5'-TGTGAGGGGTGGCAGAATG-3'
ZWINT	5'-TCTGGCGGAGGTTTCT-3'	5'-GCTGCTGGGGTTTATCAT-3'
SKP2	5'-CCCAGGAACTGCTCTCAAA-3'	5'-CTGCGGACAATCACAAAGT-3'
YWHAB	5'-CTGGGGAGGGAGAGAACTA-3'	5'-GGCTGAGGCTGTGAAAAA-3'
BIRC3	5'-CCAAGTGGTTTCCAAGGTGTG-3'	5'-TCATCTCCTGGGCTGTCTGA-3'
PRIM	5'-AATATGGACCCTGGCTGGAG-3'	5'-GCACAGATATGCGACCTGTT-3'
STAT1a	5'-CGACAGTATGATGAACACAGTA-3'	5'-AGAGTAGCAGGAGGGAATCA-3'
STAT1b	5'-ACCAGAGCCAATGGAACTT-3'	5'-CATGTCACTCTTCTGTGTTCA-3'
POLA1	5'-GCGACGACTCTCTGTCAGATT-3'	5'-TCTTTCTAGGGCTTCTTGGCG-3'
SREBF1	5'-GGCACCGAGAGCAGAGATGGC-3'	5'-GGAGACGAGCACCAACAGCCC-3'

2.3 Results

2.3.1 Expression of LXR Isoforms in Pancreatic Cancer Cells and Clinical Samples

Before characterizing the effect of LXR ligands on pancreatic cancer cell biology, which we hypothesize will restrain proliferation-related processes; we first examined LXRα and LXRβ expression in biopsies of human pancreatic cancer and PDAC cell lines. Immunohistochemical staining of LXRβ in human samples demonstrated nuclear immunoreactivity in normal pancreatic ducts (Figure 2.1A). Nuclear and cytoplasmic LXRβ immunoreactivity was detected in PDAC samples (Figure 2.1B-C), suggesting altered localization of LXRβ in these cancerous samples. Comparatively, LXRβ expression was barely detectable in a pancreatic adenoma clinical sample (Figure 2.1D). Immunostaining for LXRα was not detectable both in normal ducts (Figure 2.1E) and in a PDAC sample (Figure 2.1F). These results suggest that LXRβ is the main isoform present in pancreatic ductal epithelial cells and its abnormal localization is evident in PDAC patient tissues.

For functional studies, BxPC-3, MIA-PaCa-2, and PANC-1 PDAC cell lines were chosen for characterization because they exhibit different invasive, proliferative, and angiogenic potential [157]. Western results indicate that LXRβ was detected in BxPC-3 and MIA-PaCa-2 and PANC-1 cells, although expression levels were the lowest in the PANC-1 cells (Figure 2.1G). Consistent with our observations in clinical samples, LXRα was not detected in PDAC cell lines (Figure 2.1H). LXR agonist GW3965 also activated expression of ABCA1, a known LXR target gene [55], in all three cell lines (Figure 2.11). These findings indicate that LXRβ is expressed and functional in PDAC cells.

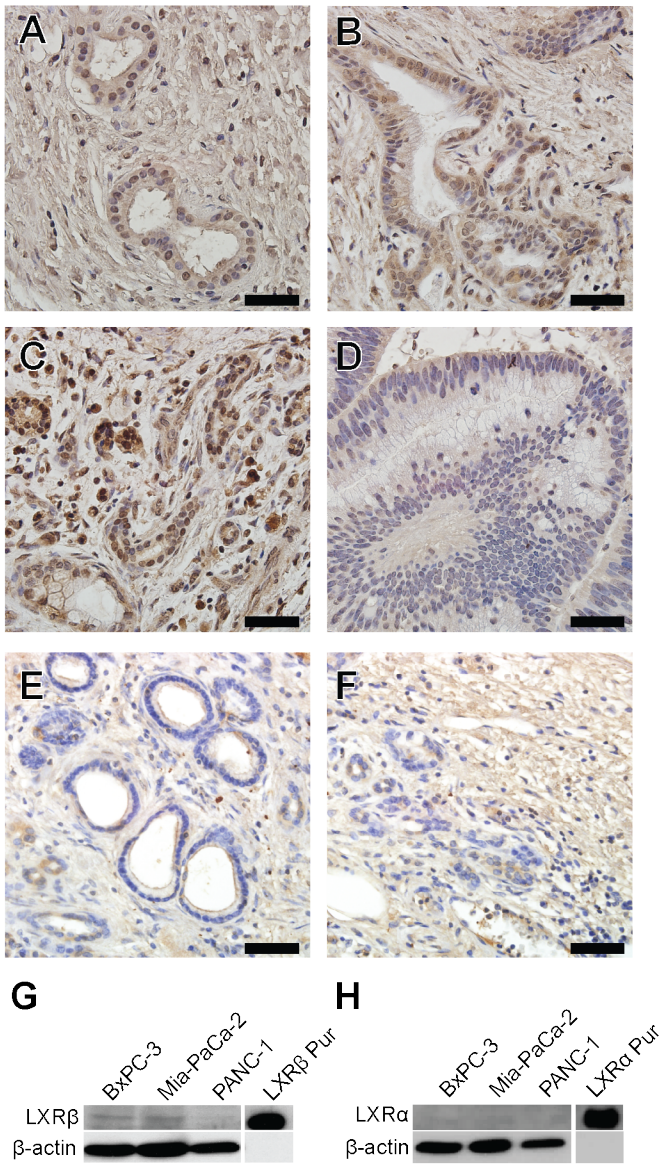


Figure 2.1. LXRβ is the main LXR isoform expressed in pancreatic cancer samples and in three pancreatic adenocarcinoma cell lines.
(A) LXRβ was detected in the nuclei of normal pancreatic ductal epithelial cells (female, age 59). (B) (C), LXRβ positive immunoreactivity was evident in both the cytosol and the nuclei of neoplastic cells of patients with pancreatic adenocarcinoma (male, age 59 and female, age 65 respectively. (D), LXRβ expression was undetectable in the pancreatic adenoma sample (female, age 59). (E), (F) LXRα immunoreactivity is not detectable in normal ductal epithelial cells (female, age 59) and in pancreatic adenocarcinoma (male age 65). (G), LXRβ is expressed in BxPC-3, Mia-PaCa-2, and PANC-1 cells. H, LXRα is not expressed in PDAC cell lines. Scale bar=50μM.

2.3.2 Anti-proliferative Effects of LXR Ligands

To determine the effects of LXR ligands on PDAC cell proliferation, cells were treated with synthetic LXR agonist GW3965 and live cells were quantified using trypan blue exclusion assays. BxPC-3 (Figure 2.2A), MIA PaCa-2 (Figure 2.2B), and PANC-1 (Figure 2.2C) cell proliferation was significantly inhibited by GW3965 treatment. At 72 hours, cell numbers were significantly lower in treated cells as compared to vehicle treated controls for all three cell lines. Titration curve experiments showed a dose-dependent inhibition of cell proliferation in all three cell lines. EC50 calculations indicated that BxPC-3 and MIA-PaCa-2 exhibited the greater GW3965 sensitivity (10.10 μM in BxPC-3 and 11.33 in MIA-PaCa-2), and PANC-1 cells were the least sensitive (13.66 μM). Additional studies using tetrazolium salt reduction assays further confirmed that GW3965 suppresses the growth of PDAC cell lines in a dose-dependent manner (Figure 2.2D). All three cell lines showed statistically significant drops in cell proliferation as measure by MTS reduction assays at 5 and 10 μM GW3965 for 72 hours as compared to vehicle-treated controls(***P-Val < 0.001). Clonogenic assays were also employed to evaluate the effects of long-term LXR ligand treatment on cell proliferation and colony formation. Activation of LXR using GW3965 strongly inhibited colony formation in each cell line (Figure 2.2E-F). Inhibition was dramatic and statistically significant at 5 and 10 μM GW3965 (***P-Val < 0.001 in all three cell lines). Colony formation was inhibited by over 95% in all three PDAC cell lines when treated with 10 μM GW3965 (Figure 2.2F). These findings suggest that LXRs are involved in PDAC cell proliferation and targeting LXRs with ligands perturb their normal functions in cell proliferation. To test this hypothesis and to determine the role of LXRs in mediating the effects of the ligands, we knocked down LXRα and LXRβ expression using small interfering RNAs (siRNAs). Transfection of PDAC cells reduced LXR expression 50-80% as compared to the controls (3.3A). Knockdown of LXRα had no effect on cell proliferation or response to treatment with the GW3965 ligand (Figure 2.3B). On the other hand, knockdown of LXRβ expression significantly reduced cell proliferation, even in vehicle treated cells, and ligand treatments following gene knockdown did

not further reduce cell proliferation. These results indicate that LXRβ is required for PDAC cell proliferation and response to LXR ligand treatment.

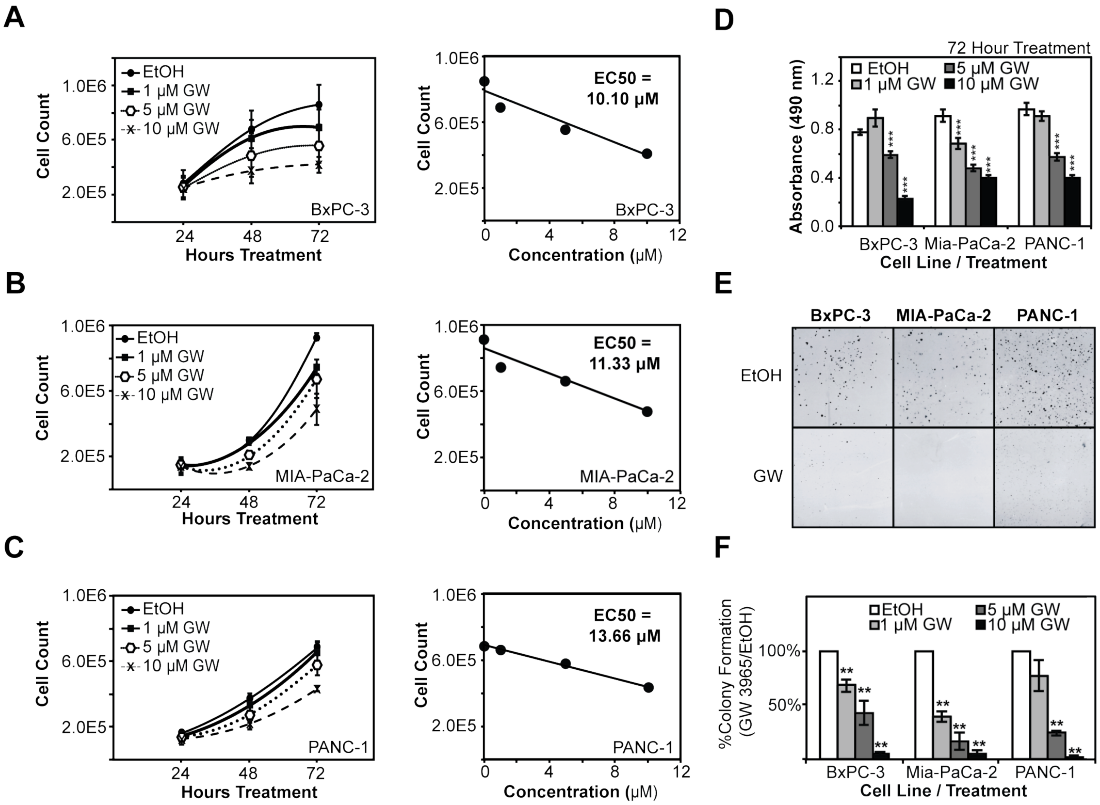


Figure 2.2. LXR agonists block cell proliferation and colony-formation in pancreatic cancer cells.

(A), (B), (C), PDAC cells (BxPC-3, Mia-PaCa-2, and PANC-1 cell lines, respectively) show dose-dependent decreases in cell proliferation upon treatment with increasing GW3965 concentrations. EC50 calculations indicate that BxPC-3 and Mia-PaCa-2 cells are more sensitive to ligand treatment than PANC-1 cells. D, Results from MTS assays, a separate measure of overall cell metabolic rate and indirect measurement of cell proliferation, demonstrate a dose-dependent drop in overall metabolism in cells treated with increasing concentrations of GW3965. (E), Colony-formation ability in all three cell lines was blocked by GW3965 treatment. (F), Colony formation of GW3965 treated cells was quantified relative to vehicle-treated controls.

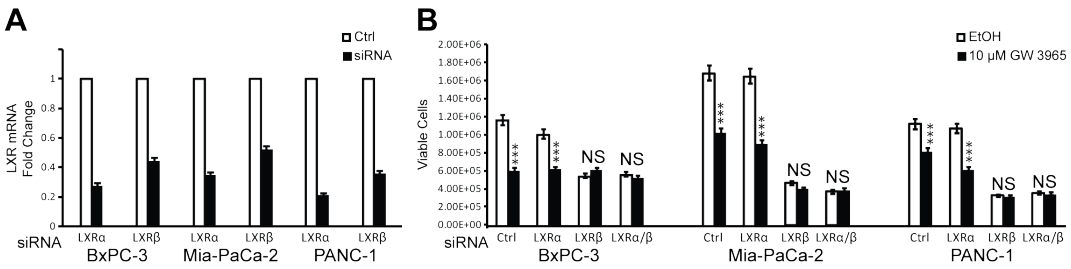


Figure 2.3. Knockdown of LXRβ expression blocks PDAC cell proliferation and response to LXR ligand treatment.

(A), Knockdown of LXRα and LXRβ expression was validated by quantitative PCR. Expression data were normalized to 36B4 ribosomal gene transcript levels. (B), The effect of LXR knockdown on PDAC cell proliferation was quantified by cell counts following trypan blue exclusion assays.

To examine whether the observed antiproliferative effects were due to the specific synthetic agonist used in previous studies, we treated PDAC cells alternatively with the T0901317 ligand. Treatments with T0901317 blocked proliferation in two cell lines, BxPC-3 and Mia-PaCa-2, but not PANC-1 (Figure 2.4). T0901317 inhibited BxPC-3 and MIA-PaCa-2 proliferation by 40.2% and 54.2%, respectively, when compared to vehicle, and the differences are statistically significant (***p-val < 0.001). PANC-1 cell proliferation was inhibited 15.3%, but the effects were not statistically significant. To mitigate potential off-target effects posed by higher ligand doses, we treated PDAC cells at a titration of lower concentrations for longer time periods than those demonstrated in 3. 1 and Figure 2.2. Lower concentrations of drug were only slight in their effect, albeit reproducible. For 1 μM treatments of GW 3965, BxPC-3 proliferation was inhibited 10.2% (p-val = 0.01), MIA-PaCa-2 growth was inhibited by 11.2% (p-val = 0.02), and PANC-1 growth slowed by 16.9% (p-val < .001) (Figure 2.5A-C). Despite a response by PANC-1 at significantly lower concentrations of drug, there was never the precipitous drop observed in BxPC-3 and MIA-PaCa-2 at higher concentrations, suggesting that PANC-1 is able to uncouple of the antiproliferative effect of GW 3965 over longer time periods. A similar long-term titration was performed using T0901317. The titration curves presented with a bimodal pattern suggesting off-target effects depending on the concentration of ligand used (Figure 2.5D-F). Similarly to GW 3965, T0901317 was maximally efficacious at 10 μM concentrations in all three cell lines. These findings suggest that there are ligand- and cell type-specific effects of LXR activation in PDAC cells.

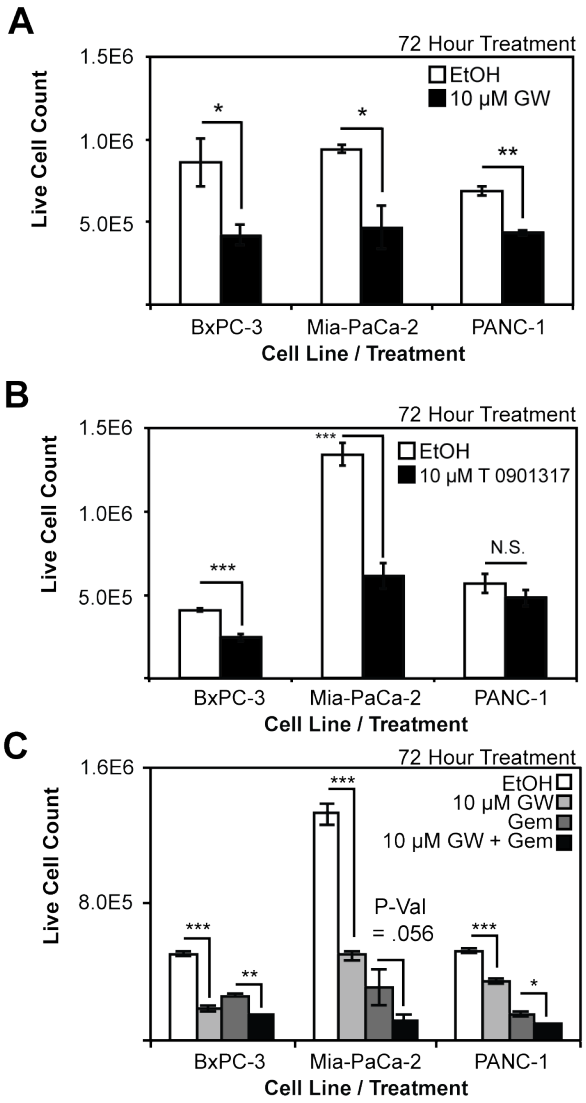


Figure 2.4. Co-treatment of pancreatic cancer cells with LXR ligands and gemcitibine reveals additive antiproliferative effects.
(A) Cell proliferation is blocked in BxPC-3, MIA-PaCa-2, and PANC-1 cell lines upon treatment with 10 μM GW 3965. (B) LXR agonist T0901317 blocks proliferation in BxPC-3 and MIA-PaCa-2 cells, but is unable to block cell proliferation in PANC-1 cells. (C) GW3965 and gemcitibine block proliferation in all three pancreatic cancer cell lines and are additive in their inhibition of proliferation when administered concomitantly.

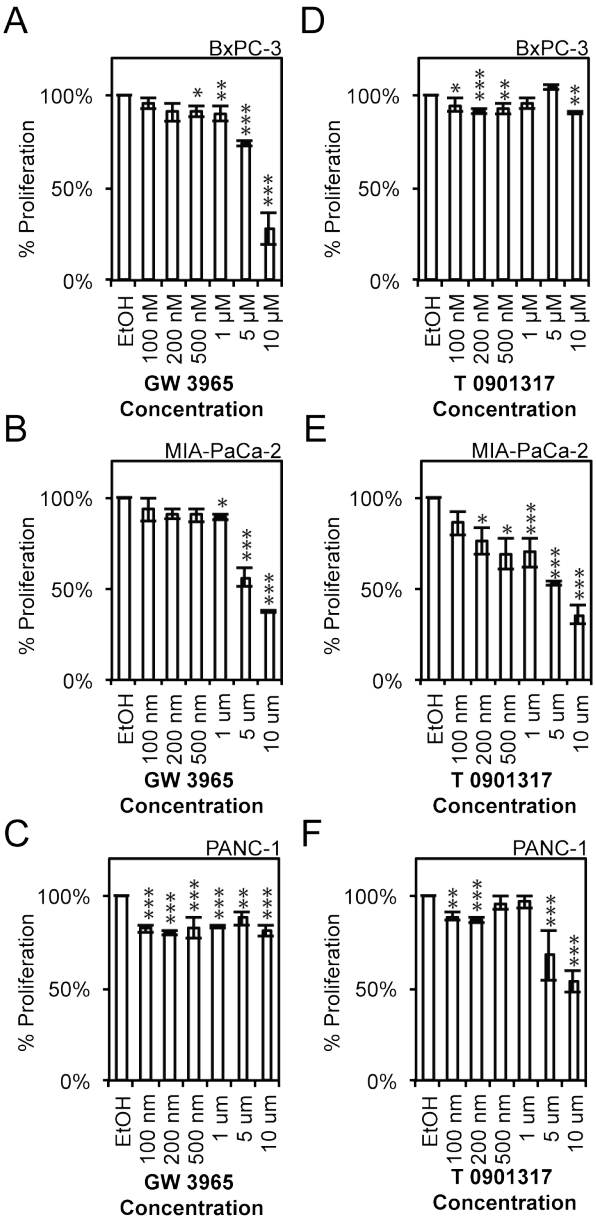


Figure 2.5. LXR ligands GW 3965 and T 0901317 have subtle effects on cell proliferation at lower concentrations and are concordant directionally with effects observed at higher concentrations.

(A) BxPC3, (B) MIA-PaCa-2, and (C) PANC-1 titrations with varying concentrations of GW 3965. (D) BxPC3, (E) MIA-PaCa-2, and (F) PANC-1 cells treated with T 0901317. Cells were treated for 1 week to more adequately resolve subtle differences in cell proliferation beween treatments. Cell proliferation is measured by MTS assay.

After demonstrating the antiproliferative effects of LXR ligands, we then compared their effects on PDAC cells to gemcitabine, a nucleoside analog chemotherapeutic with severe side effects. Cells were treated with vehicle, GW3965, gemcitabine (20 nM for BXPC-3, and 40 nM for MIA-PaCa-2 and PANC-1 cells), or combination of GW3965 and gemcitabine. Interestingly, GW3965 cooperated with gemcitabine to block proliferation in three pancreatic cancer cell lines to a greater extent than any treatment by itself. As expected, gemcitabine treatments inhibited proliferation in BxPC-3 by 49%, MIA-PaCa-2 by 77%, and PANC-1 cells by 71%; and the effects are significantly different when compared to vehicle (***p-val < 0.001) (3.4C). Co-administration of GW3965 and gemcitabine blocked proliferation in BxPC-3, MIA-PaCa-2, and PANC-1 cells by an additional 21.8%, 13.9%, and 10.5% respectively when compared to gemcitabine alone (*p-val < 0.05 in BxPC-3 and PANC-1 cells, P-Val = 0.056 in MIA-PaCa-2 cells). A longer-term experiment (7 days) was performed to assess combinatorial effects of GW 3965 and a gemcitabine titration on cell proliferation, while determining the effect of a lower dose of GW 3965 on response to gemcitabine (Figure 2.6). These results demonstrate that gemcitabine is exceptionally antiproliferative, and that the additive effects of GW 3965 observed in Figure 2.4C are directionally consistent with Figure 2.6 and statistically distinguishable at lower concentrations of gemcitabine (1 nm), albeit insubstantial. These results suggest the possibility of future studies evaluating the effectiveness of GW3965 and gemcitabine as a combination therapy.

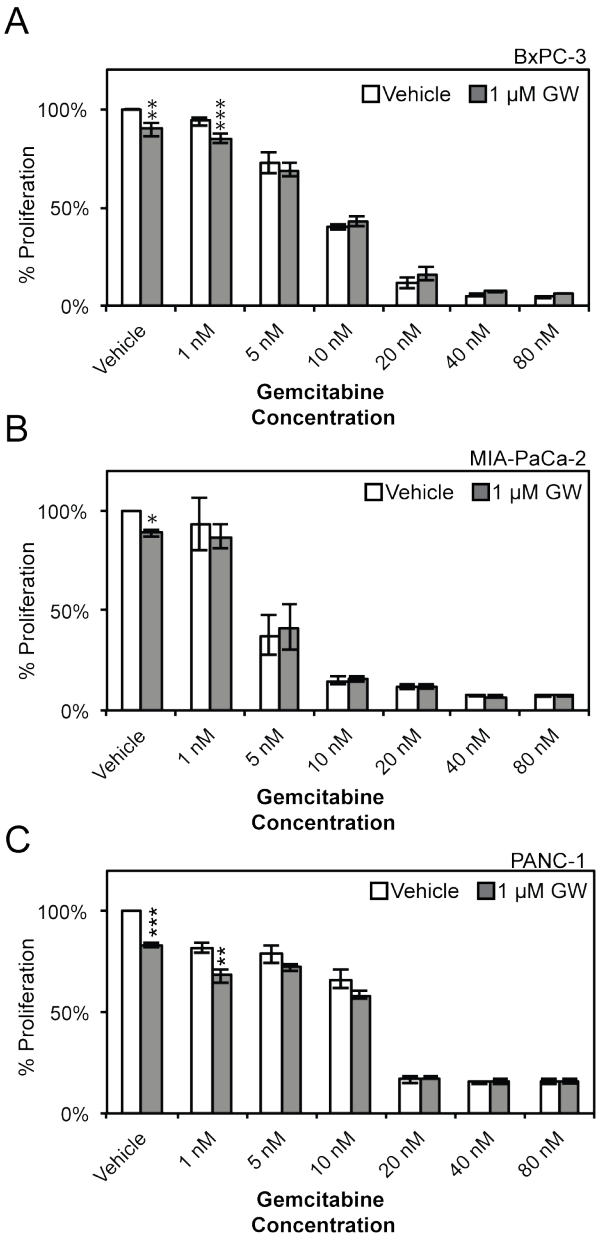


Figure 2.6. Gemcitabine titrations in PDAC cell lines are modulated by a low dose of GW 3965.

(A) BxPC3, (B) MIA-PaCa-2, and (C) PANC-1 cells treated with increasing concentrations of gemcitabine. Subtle effects on gemcitabine response are only observed at very low concentrations of gemcitabine and are only statistically significant in BxPC-3 and PANC-1 cells. Cells were treated for 1 week to more adequately resolve subtle differences in cell proliferation beween treatments. Cell proliferation is measured by MTS assay.

2.3.3 Effects of LXR Ligand Treatment on Cell Cycle Progression

Functional assays revealed that LXR ligand treatment blocked proliferation of PDAC cells. To better understand the mechanics of the antiproliferative effect, cell cycle analysis was performed following agonist treatment. Flow cytometry analysis revealed an additional 15.0% of BxPC-3 cells, 9.6% of MIA-PaCa-2 cells, and 8.4% of PANC-1 cells in G1/G0 phases of the cell cycle when treated with GW3965 (Figure 2.7A), and a corresponding 12.0% decrease of BxPC-3 cells, 9.9% of MIA-PaCa-2 cells, and 9.0% of PANC-1 in cells in S/G2/M phases of the cell cycle (Figure 2.7B). These changes are statistically significant (P-Val < 0.001). Bromodeoxyuridine (BrdU) incorporation experiments showed a decrease in DNA synthesis by 12.9%, 27.0%, and 21.0% in BxPC-3, MIA-PaCa-2, and PANC1 cells respectively (Figure 2.7C) (****p*-val < 0.001). Representative histograms for BxPC-3 (Figure 2.7D), MIA-PaCa-2 (Figure 2.7E), PANC-1 (Figure 2.7F) demonstrate a qualitative increase in G1 cells and a decrease in G2/M cells in GW 3965 treated cells. Similarly, BrdU-incorporation density plots for each cell line demonstrate a qualitative decrease in BrdU+ cells upon treatment with GW 3965 (Figure 2.7G). Taken together, these findings demonstrate that LXR agonists inhibited PDAC cell proliferation by blocking cell cycle progression. To further uncover potential mechanisms of this effect on the cell cycle, we determined protein expression of cell cycle mediators known to be regulated by LXR ligand treatment in breast cancer cell lines[55]. Of the cell cycle regulators regulated in breast cancer cell lines, Skp2 is the only gene to respond in a similar manner between breast and pancreatic cancer cell lines. Western analysis confirms that Skp2, an oncogene, is downregulated 1.6 fold in BxPC-3 cells, 6.4 fold in MIA-PaCa-2 cells, and unchanged in PANC-1 cells when treated with 5 μM GW 3965(Figure 2.8A-D) (**p*-val < 0.05 in BxPC-3 and MIA-PaCa-2 cells, whereas *p*-val = 0.43 in PANC-1 cells). A mechanism tying LXR directly to SKP2 transcriptional regulation, however, is not likely, as transcription levels do not correspond to protein levels upon treatment with GW3965 (Figure 2.9). This suggests that other, more upstream regulators are responsible for the observed antiproliferative effect. We specifically examined the expression of EGFR, a factor overexpressed in pancreatic cancers and the only non-chemotherapeutic marker that has

been successfully targeted in the treatment of PDAC [77]. EGFR is repressed 1.45 and 1.88 fold in the more sensitive BxPC-3 and MIA-PaCa-2 cell lines upon treatment with 5 μM GW3965 (Figure 2.8B-C), and is statistically significant when compared to vehicle. This decrease in EGFR expression was not observed in PANC-1 cells, possibly due to their lesser sensitivity to LXR ligands (Fold Change: +1.24, *p*-val = 0.19) (Figure 2.8D). Decreases in EGFR expression levels in BxPc-3 and MIA-PaCa-2 coincide with decreases in phospho-EGFR (Tyr1173). Phospho-EGFR levels decrease 1.93 fold in BxPC-3 and 1.65 fold in MIA-PaCa-2 (Figure 2.8A-C). To further assess the downstream effects of a downregulated EGFR in BxPC-3 and MIA-PaCa-2, ERK (p44/p42) and phosphorylation status were detected. No statistically significant changes to either total ERK or phospho-ERK were observed, suggesting that a downregulated EGFR could effect change on cell proliferation through other mechanisms. Taken together, these findings suggest that GW3965 inhibits transit of PDAC cells through the cell cycle, possibly by regulating key proteins that are responsible for G1-S transition and growth factor receptors that are heavily involved in regulating cell migration, proliferation, and survival [77,158].

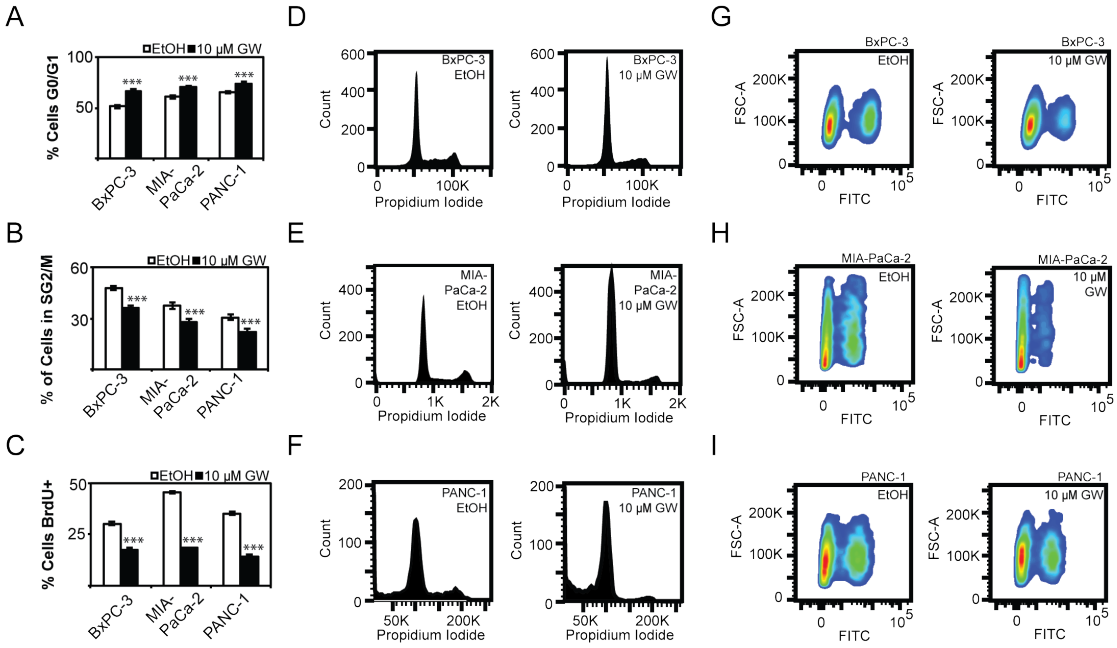


Figure 2.7. LXR agonists block pancreatic cancer cell progression through the cell cycle. (A), GW3965 treatment arrests a significant proportion of the cells in the G1/G0 stage of the cell cycle as measured by propidium iodide staining and flow cytometry. (B), Fewer cells are found in S, G2, or M phases following ligand treatment. (C), BrdU-pulse analysis demonstrates that GW3965 treatments reduce transit through the S-phase of the cell cycle. (D), (E), (F) Representative cell cycle analysis diagram of BxPC-3, MIA-PaCa-2, and PANC-1 cells respectively. (G), (H), (I) Density plot depicting the number of cells staining for BrdU as a measure of S-phase transit in BxPC-3, MIA-PaCa-2, and PANC-1 cells.

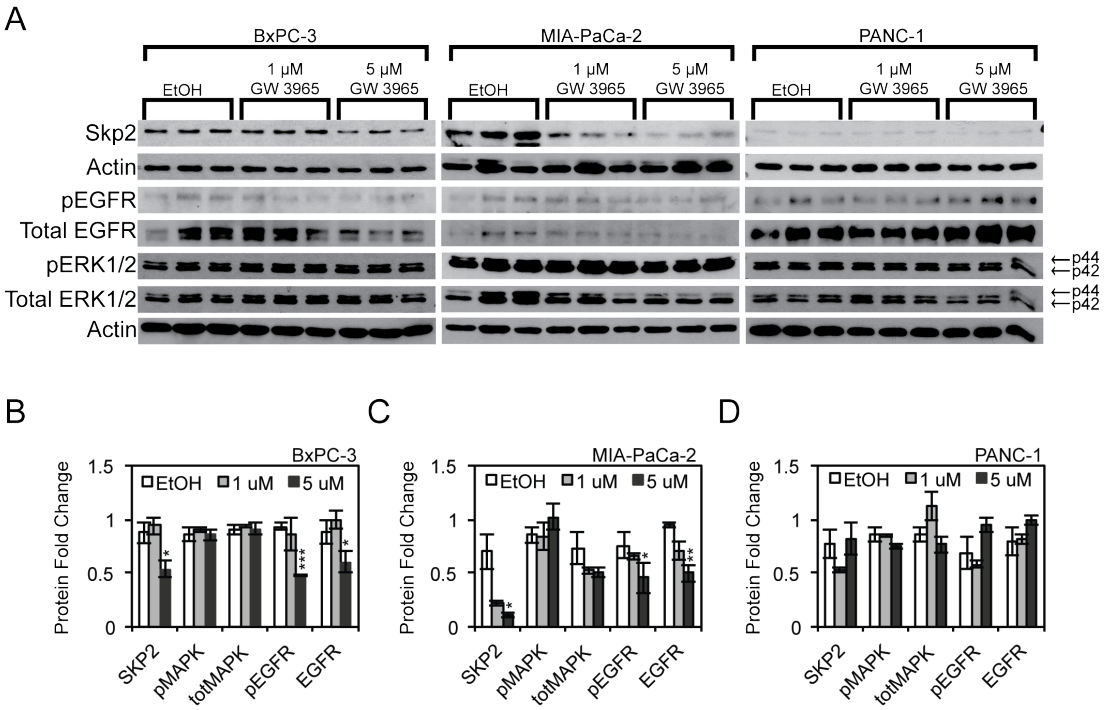


Figure 2.8. GW 3965 down-regulates oncogenes involved in cancer progression.
(A), GW3965 treatment downregulates Skp2 and EGFR protein levels in BxPC-3 and MIA-PaCa-2 cells. Downregulation of EGFR was concomitant with a downregulation of its own phosphorylation in BxPC-3 and MIA-PaCa-2 at 5 uM GW 3965. ERK1/2 and its phosphorylation were not statistically different in any of the cell lines. (B), (C), (D) Densitometric quantification of SKP2, EGFR, Phospho-EGFR, ERK1/2, and Phospho-ERK1/2 upon treatment with GW3965. Samples were normalized to actin controls.

A

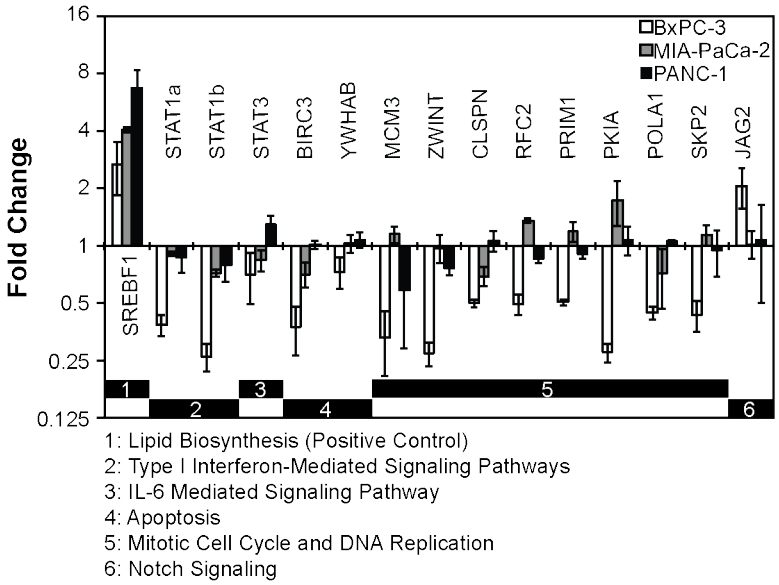
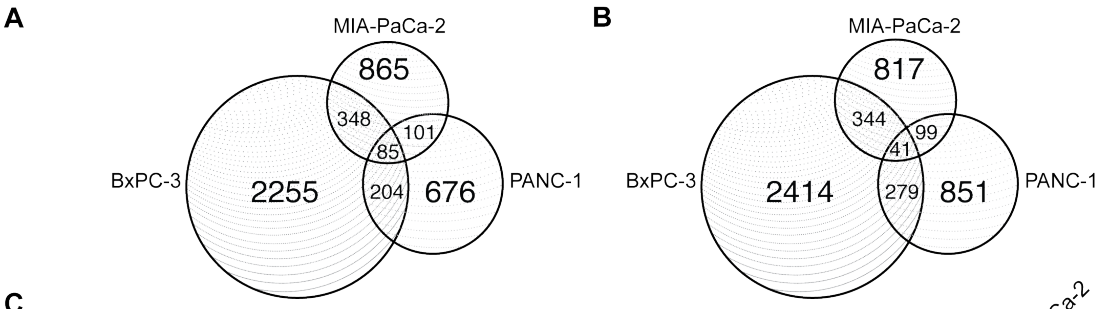


Figure 2.9. Validation of microarray on pancreatic cancer cell lines treated with LXR agonists reveal potential mechanisms of cell proliferation inhibition.
(A) All cell lines share upregulated lipid biosynthetic regulators (Group 1). Type-I interferon mediated pathways are downregulated in three PDAC cell lines, but most robustly in BxPC-3 cells (Group 2). Genes that regulate apoptosis are downregulated strongly in BxPC-3 (Group 4). Mitotic cell cycle is downregulated most consistently in BxPC-3 cells, but also in MIA-PaCa-2 and PANC-1 cells (Group 5).

2.3.4 Microarray Analysis of Effects of LXR Ligands on Gene Expression

Activation of LXR, a ligand-dependent transcription factor, is expected to directly or indirectly alter the expression of genes involved in proliferation-related pathways in pancreatic cancer cells. Microarray analysis of GW3965 responsive genes in three PDAC cell lines revealed common and cell line-specific responses. BxPC-3, MIA-PaCa-2, and PANC-1 cell lines showed distinct differences in the total number of up-regulated genes, numbering 2255, 865, and 676 in each respective cell line (Figure 2.10A). Of these, only 85 had concordant responses in all three cell lines. A similar distribution of down-regulated responsive genes was noted in the three cell lines, with the most robust response observed in BXPC-3 cells, with 41 genes commonly down-regulated in all three cell lines (Figure 2.10B). Gene ontology and pathway analysis of responsive genes showed that ligand treatment up-regulated genes involved in lipid metabolic, triglyceride biosynthetic, and long-chain fatty-acyl-CoA biosynthetic processes, including previously identified LXR target genes (Figure 2.10C). This is consistent with LXR's known roles in cholesterol and lipid metabolism in other tissues [55]. Commonly down-regulated genes include those that regulate cellular response to viral infection (Figure 2.10C). Down-regulated pathways that were shared between BxPC-3 and PANC-1 cell lines regulate cell cycle progression and DNA replication, while down-regulated pathways shared between BxPC-3 and MIA-PaCa-2 regulate modulators of immune response, such as the innate immune response and type I interferon-mediated pathways (Figure 2.9). Pathways responsible for cytoskeleton organization, apoptosis, and inflammatory-related pathways are also differentially expressed, which suggests that LXR ligands may regulate other cancer-related processes such as metastasis or cell survival in models of PDAC. These results indicate that activation of LXRs using LXR ligands result in dramatic antiproliferative and anticlonogenic effects in PDAC cells in general, but the underlying mechanisms of action appear to be varied.



C

	Up-Regulated Gene Ontology	Down-Regulated Gene Ontology	BxPC-3	MIA-PaCa-2	PANC-1
1	(1) Mitotic cell cycle, (2) Cell cycle, (3), Cell division, (4) Mitosis, (5) Metabolic Process	(1) Metabolic process, (2) Transcription, DNA-dependent, (3) Apoptosis, (4) Transport, (5) Positive regulation of protein phosphorylation		⊙	
2	(1) Biosynthesis of cholesterol, (2) Mevalonate pathway, (3) Adipocytokine signalling, (4) Insulin action (5) Branched chain amino acids metabolism	(1) Transcription, DNA-dependent, (2) Regulation of transcription, DNA-dependent, (3) Response to endoplasmic reticulum stress, (4) Cell redox homeostasis, (5) Fatty acid oxidation		⊙	⊙
3	(1) Metabolic process, (2) Multicellular organismal development, (3) Regulation of apoptosis, (4) Interspecies interaction between organisms, (5) Response to hypoxia	(1) Transcription, DNA-dependent, (2) Phosphorylation, (3) Metabolic process, (4) Protein transport, (5) GTP catabolic process			⊙
4	(1) Metabolic process, (2) PDGF receptor signaling pathway, (3) Response to hypoxia, (4) Negative regulation of cell proliferation, (5) Response to stress	(1) Cell cycle, (2) Mitotic cell cycle, (3) DNA repair, (4) DNA replication, (5) S phase of mitotic cell cycle	⊙		⊙
5	(1) Metabolic process, (2) Transcription, DNA-dependent, (3) Transport, (4) Phosphorylation, (5) Protein transport	(1) Metabolic process, (2) Mitotic cell cycle, (3) Transcription, DNA-dependent, (4) DNA repair, (5) Cell cycle	⊙		
6	(1) Metabolic process, (2) Apoptosis, (3) Transport, (4) Cellular lipid metabolic process, (5) L-serine biosynthetic process	(1) Response to virus, (2) Type I interferon-mediated signaling pathway, (3) Cytokine-mediated signaling pathway, (4) Metabolic process, (5) Innate immune response	⊙	⊙	
7	**(1) Lipid metabolic process, (2) Lipid biosynthetic process, (3) Cellular lipid metabolic process, (4) Triglyceride biosynthetic process, (5) Long-chain fatty-acyl-CoA biosynthetic process**	**(1) Response to virus, (2) Terminal button organization, (3) Transmembrane transport, (4) Late viral mRNA transcription**	⊙	⊙	⊙

Figure 2.10. Microarray analysis of pancreatic cancer cell lines treated with LXR ligands defines common and cell line-specific effects on gene networks.
(A), (B), Venn diagrams of up-regulated and down-regulated genes (1.1 fold change cutoff) after treatment with GW 3965 for 72 hours. These cell lines show common and cell-line specific transcriptomic responses to ligand treatment. (C), Microarray analysis of upregulated genes show that all cell lines share up-regulation of lipid metabolic, glucose metabolic, and cell proliferation responses. All cell lines downregulate pathways that regulate response to viral infection, transmembrane support, as well as viral mRNA transcription. BxPC-3 and PANC-1 cells also downregulate cell cycle and DNA replication machinery.

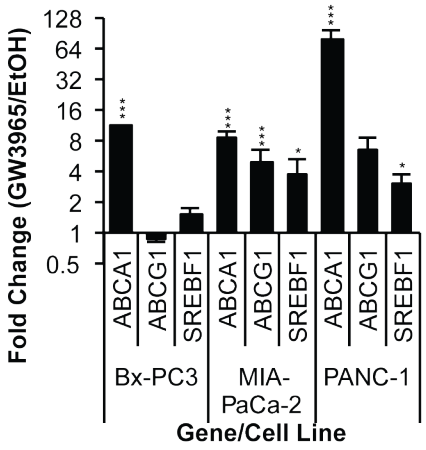


Figure 2.11. LXR ligand treatment induces expression of known LXR target genes in PDAC cell lines.
(A) ABCA1 is upregulated in all three cell lines by GW 3965 treatment, while SREBF1 is strongly upregulated in MIA-PaCa-2 and PANC-1 cells. ABCG1 is upregulated in MIA-PaCa-2 cells only. (P-Val * <0.05, **<0.025, ***<0.01).

2.4 Discussion

In this study, we tested the hypothesis that LXR activation with synthetic agonists can halt the proliferation of pancreatic cancer cells. Before assessing the effects of LXR ligands in PDAC cells, we first demonstrated that LXRβ is the main LXR isoform detectable in human pancreatic ductal epithelial cells, as LXRα is not detectable in human normal pancreatic ducts (Figure 2.1E), pancreatic adenocarcinoma (Figure 2.1F), or in PDAC cell lines (Figure 2.1H). Our studies uncovered variation in the expression levels of LXRβ in PDAC cell lines, as well as differences in sub-cellular localization of LXRβ in PDAC primary samples (Figure 2.1A-D). Unliganded LXRβ has been shown to be partially exported from the nucleus to the cytoplasm [159], suggesting that there are either differences in endogenous activating ligands in clinical samples, or variable regulation of mechanisms involved in nuclear import/export where cytoplasmic staining of LXRβ is stronger (Figure 2.1B-C). Differential localization of LXRβ proteins in clinical samples suggests that LXRβ may be suppressed in malignant cells by exclusion from the nucleus, but a more comprehensive study is needed to determine whether cytoplasmic staining of LXRβ is associated with disease progression and patient survival.

Functional assays clearly demonstrated that activation of LXRs by GW3965 in PDAC cell lines resulted in dramatic decreases in proliferation as measured by trypan blue exclusion assays (Figure 2.2A-C). Calculations of the EC50 for individual cell lines revealed that BxPC-3 and MIA-PaCa-2 cells were more sensitive to ligand treatment than PANC-1 cells. This difference in response may be due to the lower expression of LXRβ in this cell line when compared to others (Figure 2.1E). PANC-1, therefore, may represent a more refractory cell line. This notion is further supported by clonogenic assays, which demonstrate reduced PANC-1 sensitivity to ligand treatment (Figure 2.2F), as well as complete PANC-1 insensitivity to another LXR agonist T0901317 (Figure 2.4B). T0901317 is a promiscuous binder of other nuclear receptors, such as farnesoid X and RAR-related receptors, which may explain why the effects of GW3965 (Figure 2.4A) are not completely recapitulated by T0901317 [152,160-162]. The diverging effects of

alternative LXR ligands may also be attributable to differences in LXRβ expression levels, metabolism of the compound, or epigenetic modifications that potentiate alternative mechanisms.

Recent advances in the treatment of PDAC pair the existing pair standard-of-care chemotherapeutic gemcitabine with an EGFR tyrosine kinase inhibitor such as erlotinib. Before this, gemcitibine was the first therapeutic that was able to extend survival since it replaced 5-fluorouracil as the preferred chemotherapeutic agent in 1997 [163]. This suggests that combination therapy including gemcitabine is one of a limited set of viable strategies in the development of therapeutics for pancreatic cancer. We demonstrate here that gemcitabine concomitant with GW3965 may be superior to either treatment by itself in the three pancreatic cancer cell lines (Figure 2.4C).

To understand the cellular mechanisms underlying cancer cell proliferation inhibition by LXR agonists, we utilized flow cytometry to quantify changes in the cell cycle in PDAC cells. GW3965 treatment arrested PDAC cells in the G1/G0 phase of the cell cycle (Figure 2.7A,B). It also strongly inhibited BrdU incorporation, a measure of cell S-phase transition (Figure 2.7C). These data show that LXR activation by synthetic agonists results in cell cycle arrest, but does not indicate mechanisms linking LXR's known role as a transcription factor to its antiproliferative effect. Here we show that Skp2, an oncogene previously shown to be down-regulated in ligand treated breast cancer cells, is down-regulated in two sensitive PDAC cell lines as a consequence of GW3965 treatment (Figure 2.8B-C). Skp2 is known to regulate c-Myc transactivation and ubiquitination, and regulates the turnover of other cell cycle regulatory units in maintenance of normal G1-S transition [161,162]. LXR ligand treatment also down-regulates epidermal growth factor receptor (EGFR) in two cell lines (Figure 2.7B-C), raising questions about LXRs and their effects on apoptosis and migration in PDAC, as EGFR is integrally linked to these pathways. Interestingly, activation of LXR using GW3965 has been shown to sensitize glioblastoma cells expressing EGFR splice variant (EGFRvIII) to apoptosis in *in vivo* models of glioblastoma [164]. We did not observe increases in cell death, however, following ligand treatment (Figure S5).

These results suggest that LXRs are integrally tied to machinery regulating cell cycle progression and growth factor signaling.

It has been posited that gene networks involved in cholesterol and fatty-acid metabolism are tied to LXR's emerging roles in cancer cell growth [55,165]. Activation of LXR leads to strong up-regulation of SREBF1 (sterol regulatory element-binding protein 1c) in breast, colon, pancreatic cancer cells (Figure 2.10C, 3.11), and is a regulator of lipogenesis and glucose metabolism [55,166]. Knockdown of SREBF1 protein in breast cancer cells, however, did not block cell proliferation inhibition by LXR agonists [55]. Interestingly, published studies linked sterol metabolic pathways to proliferation of T-cells through ABCG1 in normal T cell physiology. Inactivation of this transporter prevented LXRβ-mediated inhibition of proliferation in T cells [165]. The addition of low-density lipoproteins to the medium of T cells did not interfere with cell proliferation. These findings suggest that cholesterol may not only be a constituent of the cell membrane, but may be dynamically regulated intracellularly as a component of cell cycle progression controlled by cholesterol transporters and other factors. Despite promising potential leads into mechanisms potentially regulated by LXR agonists, more work needs to be done in pancreatic tissues to elucidate how this effect is achieved.

Mechanistically, our microarray study showed both concordant and discordant gene responses in three PDAC cell lines. Up-regulated genes (Figure 2.10A) are enriched for those known to function in cholesterol and fatty acid metabolism (Figure 2.10C), whereas down-regulated genes (Figure 2.10B) were less concordant, and function in pathways that regulate response to viral infection (Figure 2.10C). Differences between cell lines in these responses can be attributed to variation in epigenetic modifications that potentiate LXR activity at response elements after ligand stimulation. Genome-wide microarray studies in breast cancer cells show that up-regulated genes tend to regulate cholesterol and fatty acid metabolism, whereas down-regulated genes function in DNA replication and cell cycle programs. Specifically, treatments with LXR agonists down-regulated the expression of E2F2, a member of the E2F family of transcription factors. However, knockdown of E2F2 in breast cancer cell lines only blocked

proliferation in ER+ cell lines which suggests that mechanisms of cell proliferation inhibition by LXRs may be diverse in nature [56]. It is important to note that E2F2 is repressed significantly in BxPC-3 cells and not in the other two PDAC cells, and this observation suggests that mechanisms discovered in breast cancer cells may not necessarily be involved in pancreatic cancer cells. Differences in experimental design may also explain the variations noted between tissues (i.e. breast vs. pancreas). Treatment time was 72 hours for PDAC cell lines, whereas a shorter 48 hour-treatment was used for breast cell lines. Therefore, analyses in PDAC cells likely uncovered more secondary and tertiary responses to ligand treatment, and justifiably so because inhibition of cell proliferation by LXR is not thought to be a primary response. Future studies using shorter treatment times will shed light on early mechanisms underlying the effects of LXR agonists.

These initial studies demonstrated the effects of LXR ligands on cell proliferation, but more work is needed to characterize their effects on other cancer-related processes. Treatment with LXR agonists induced apoptosis in prostate cancer cell lines and xenograft models by down-regulating AKT signaling [60]. However, our data show that LXR activation in pancreatic cancer cells was solely anti-proliferative, and lacked the ability to induce apoptosis as measured by caspase-3 cleavage (data not shown).

Interestingly, LXRβ knockdown leads to a dramatic decrease in proliferation in PDAC cell lines (Figure 2.3B), which logically conflicts with the notion that LXRβ is antiproliferative. However, titration experiments using lower doses of GW 3965 to activate LXRβ have revealed a slight although reproducible effect on cell proliferation that is directionally consistent with higher doses (Figure 2.5). This suggests that the effect of GW 3965 is mediated through LXR, and the precipitous drop in proliferation observed at higher concentrations may be initiated by a cellular feedback or degradation mechanism, potentially altering both the available pool of RXR as well as available DNA binding sites in the absence of LXRβ. In spite of cursory evidence that GW 3965 acts through LXR, there are possible antiproliferative effects that could be mediated through an LXR-independent mechanism.

Additional work is needed to characterize LXR function in the context of cell motility, migration, and the unique effects of other LXR ligands on pancreatic cancer biology in both cell-based and animal models. These findings, however, indicate that LXR agonists and their derivatives warrant further study and development as potential therapeutic agents in the treatment of pancreatic cancer.

2.5 Conclusions, Context, and Future Directions

We demonstrate in these studies that LXR ligands result in dramatic antiproliferative and anticlonogenic effects in PDAC cells. LXRβ is the main isoform present in pancreatic ductal epithelial cells, and is abnormally localizated in PDAC patient tissues when compared to normal samples. Future experiments should analyze LXRβ expression levels in a larger cohort of cancers, which would allow for more quantifiable and statistical comparisons between cancerous and normal tissue. We show that LXRβ is required for PDAC cell proliferation and response to LXR ligand treatment. LXR ligand GW 3965 inhibits transit of PDAC cells through the cell cycle, possibly by regulating key proteins that are responsible for G1-S transition and growth factor receptors that are heavily involved in regulating cell migration, proliferation, and survival [77,158]. Future experiments should seek to better understand the mechanisms of LXR-mediated repression of EGFR, as a precise link has not been explored. This may allow for future combination therapies, as EGFR is a targeted marker in pancreatic cancer. Microarray studies on GW 3965 treated cells demonstrated a consistent effect of LXR ligands on fatty acid synthetic pathways, but less clear are the mechanisms of its effects of cell proliferation pathways. Future studies should utilize more comprehensive microarray experiments to look at the primary effects of the LXR ligands on gene transcription. We determined the effects of GW 3965 at 72 hours of treatment, which may be too long as these drugs are metabolized by enzymes and their responses uncoupled or attenuated. This would allow for the identification of key antiproliferative pathways targeted by the LXRs, and the development of more specific ligands to target them. Lastly, These ligands must also be tested in live animal models of pancreatic cancer. Preliminary

experiments are currently underway to test the viability of ligands as anti-cancer agents in immunocompromised mice. After this, the effect of LXR ligands on pancreatic cancer cell proliferation will be carried out on other mouse models with intact immune signaling because of the anti-inflammatory effects of the LXR ligands. Due to the limited nature of our understanding of the LXRs in cancer, more complete maps of the intersection between LXRs as metabolic regulators and the LXRs as antiproliferative agents are needed.

The LXRs may be important for signaling the nutritional status within the organism. This may prevent the use of more advantageous energy sources by the cancer cells, and may represent one mechanism whereby LXR ligands suppress cell proliferation. Because the LXRs respond to cholesterol (derivatives), which are found in lipoprotein particles, it is possible that they are able to signal ***organismal-wide depletion of fatty acid stores in circulating lipoprotein particles***. Uptake of these LDL particles in the liver results in the conversion of cholesterol into bile acids, and upregulates lipogenesis and circulating VLDL (increasing serum triglycerides). Interestingly, the receptor for LDL particles, LDLR, is downregulated by LXR ligands in glioblastoma cancer cells and HEPG2 cells [164,167]. VLDL, or lipoprotein particles that contain endogenously generated triglyceride particles, are recognized and imported by VLDL receptors in tissues throughout the body. Both LDLR and VLDLR both contain a recognition sequence of IDOL, or the inducible degrader of LDLR [167]. This degradation mechanism is activated by the LXRs to prevent further cholesterol import. As a consequence, activation of LDLR degradation with LXR ligands may also lead to the degradation of VLDLR, which would attenuate both the import of cholesterols and fatty acids into target tissues.

It has been shown that the LXRs, FXR, and the PPARs are dynamically regulated to signal the nutritional status of the organism. A better understanding of the complex interplay between these receptors may facilitate more therapeutics that target the nutrient uptake and utilization of cancer cells. These receptors are integral to physiological decisions involved in energy allocation, such as activating or suppressing de novo lipogenesis in the liver, building up or breaking down glycogen stores, or burning/storing ingested fat compounds. For instance,

PPAR isoforms regulate the oxidation (α/δ) of fatty acids, whereas PPARγ regulates fat storage in adipose tissue [1]. The LXRs activate lipogenesis in the liver. It has also been shown that FXR activates VLDLR expression in human liver cells [168]. **It is possible that LXR and FXR together are important for sensing the nutritional status of the organism.** For instance, active FXR is indicative of reabsorbed bile acids in the portal vein. Signaling that a meal was ingested and is in the process of digestion and absorption. FXR may reactivate VLDLR suppressed by LXR-mediated degradation, which would further strengthen the notion of an LXR-FXR relationship. Furthermore, FXRs are overexpressed in pancreatic cancers, which suggest that pancreatic cancers may respond more aggressively to FXR hormones circulating in the blood stream after their reabsorption in the intestine [169]. Therefore, it would be interesting to explore: 1) the relationship between LXR agonists and LDLR/VLDLR expression levels in pancreatic cancers, and 2) the regulation of VLDLR levels response to FXR ligands in pancreatic cancers. A more complete understanding of the roles of these ligands in the regulation of cancer development and progression may lead to more targeted therapies with fewer side effects.

Due to the roles of LXRs in regulating lipogenesis, there is the possibility that LXR ligands regulate cell division by modulating internal sensors of nutrient status. Cells that lack nutrients or growth factors innately activate autophagy pathways as a means to survive, which is a particularly complicated factor in cancers [170]. There is also the possibility that LXR ligands may regulate cancer autophagy and turnover of aging cellular products (due to the potential attenuation of VLDLR and LDLR or other LXR associated mechanisms). Autophagy is a complex process, and has roles in both protecting and preserving the cancer [171]. Preliminary experiments in our lab have shown an increase in the expression of autophagy markers in pancreatic cancer cells upon stimulation with LXR ligand GW 3965. Increases in autophagy may also explain decreased EGFR expression due to upregulated turnover and degradation of cellular products. Therefore, future projects will likely explore the relationships between fat (cholesterol/fatty acid) metabolic pathways and cell proliferation pathways, which will better

assess the viability of LXR ligands as cancer therapeutics in pancreatic cancer, and may provide more general mechanisms that may apply to other cancers and metabolic syndromes.

In addition to their antiproliferative effects, the LXRs attenuate inflammation in mouse models. Chronic and sporadic inflammation in the pancreas is a risk factor for the development of pancreatic cancer. These inflammatory events lead to the secretion of cytokines that are known to promote carcinogenesis, such as TGFβ, PDGF, and TNFα [172]. Inflamed tissues recruit immune mediators, which promote angiogenesis, extracellular matrix remodeling, and cell migration/metastasis. Concurrent with this is the recruitment of extensive stromal tissue around the tumor cells, which themselves secrete tumor promoting cytokines and growth factors to facilitate tumor growth and resistance to chemotherapeutics. Constitutive expression of NFκB (the transcriptional effector of TNFα) has been observed in 67% of pancreatic cancers, which results in decreased apoptosis and an altered relationship with transrepressive nuclear receptors and mediators of the immune system [45,173]. NFκB is a transcription factor that controls genes responsible for proliferation, angiogenesis, motility, and invasiveness [174]. TNFα and NFκB factors also form an autoregulatory loop, which is thought to amplify inflammatory responses [172]. Therefore, NFκB is activated in most cancers and functions as an atypical oncogene. For these reasons, a more complete understanding of the general roles of LXRs in cancer-associated inflammation is needed.

The best understood cell-based contribution of the immune system to an aggressive cancer outcome, however, is signaling that regulates macrophage phenotypes, which are classified as type M1 or M2 [174,175]. The M1 macrophage program is activated by IFN-γ and components of pathogenic machinery, and express inflammatory cytokines such as TNF-α, IL-6, IL-23, and IL-1. Conversely, M2 macrophage (very similar to tumor associated macrophages) programs are typically activated once the pathogen has been eliminated and are induced by IL-10, an anti-inflammatory cytokine, and mediate healing of tissue damaged by pathogens and aggressive M1 macrophage activities [174,176]. Future experiments should determine more precisely how the stimulation of the LXRs with ligands effects, 1) NFκB activity and inflammatory

signaling in the main tumor cells, 2) the transcriptome and phenotypes of macrophages embedded in the tumor microenvironment, and 3) regulation of the tumor associated stroma by LXR ligands. These experiments will require the use of special mouse models of pancreatic cancer where the immune signaling is intact. Syngenic tumor models or mice genetic lines that spontaneously generate pancreatic tumors are possible future directions for these sorts of experiments. KPC mice, however, conditionally express mutant p53 and Kras in pancreatic cells (Pdx1-Cre) and develop pancreatic adenocarcinomas that recapitulate traits of human pancreatic adenocarcinomas, which tend to be poorly vascularized and insensitive to gemcitabine [177]. Poor vascularization is not recapitulated by other xenograft or cell line models of pancreatic adenocarcinoma. Therefore, the KPC mouse would be the optimal model to study the immune component of pancreatic cancer. These data will hopefully yield a better understanding of the mechanisms of LXRs in pancreatic cancer in these diverse contexts, including nutrient absorption and utilization, growth factor signaling, autophagy, and the interaction with immune and inflammatory components of the cancer stroma.

3. Alcohol Regulates Genes that are Associated with Response to Endocrine Therapy and Attenuates the Actions of Tamoxifen in Breast Cancer Cells

3.1 Introduction

More than 230,000 women will develop breast cancer in the US this year, which is currently one of the most common causes of cancer deaths in American women and is the most costly of all cancers (Cancer Facts and Figures, American Cancer Society, 2014). A more complete understanding of genetic, hormonal, environmental, or behavioral factors involved in the development of breast cancer may provide more effective preventative measures as well as more targeted therapeutics. Many contributing factors are known to increase breast cancer risk, including modifiable behaviors such as alcohol consumption. Epidemiological studies have strongly linked alcohol consumption to increased breast cancer risk [147-150]. Moreover, these studies also show that breast cancer risk is positively correlated with the amount of alcohol consumed. Alcohol consumption also positively correlates with increases in breast area covered by dense parenchymal tissue and decreased β-carotene circulation, parameters which are individually known to result in increased breast cancer risk [178-181]. Furthermore, some gene product mutations (such as GSTM1) potentiate the risk for alcohol-associated cancers [182]. Given how extensive alcohol consumption is among women in the United States, and the presence of other significant public health challenges related to alcohol abuse, alcohol consumption is a key modifiable factor in the development of breast cancer.

Alcohol-associated breast cancers tend to be estrogen receptor (ER)-positive and progesterone receptor (PR)-positive [178,183-186]. Studies examining the potential effects of alcohol consumption on the amount of circulating estrogens in the body have failed to identify a consistent correlation, suggesting that alcohol likely mediates more direct effects on signaling mechanisms in the breast to promote carcinogenesis [187,188]. It has been shown that alcohol stimulates proliferation, up-regulates ERα and aromatase expression, and attenuates BRCA1 expression in ER+ cell lines [189,190]. Furthermore, it has been previously shown that alcohol

up-regulates polymerase III specific genes, and that this effect is countered by treatment with ER antagonists [191,192]. Alcohol has also been shown to increase the migration and invasion of breast cancer cell lines, which could be mediated through decreased E-cadherin expression, or up-regulated matrix metalloproteinase secretion [193,194]. Conversely, alcohol has been shown to suppress lung metastasis of 4T1.2 breast cancer cells, which are ER- [195]. These results are difficult to interpret due to the tendency of alcohol-associated cancers to be ER+/PR+. However, another study shows that alcohol increases lung metastasis of an ER+ cell line, MADB106 [196]. These experiments were performed in male rats, but demonstrate that alcohol may regulate breast carcinogenesis in an estrogen-dependent manner. Taken together, these observations suggest that the effects of alcohol are dependent on the ER machinery. ER and PR are markers of estrogen-dependent tumor growth and sensitivity to endocrine therapy with selective estrogen receptor modulators (SERMs), which block ER activity in the breast, or aromatase inhibitors, compounds that attenuate *de novo* estrogen production in breast tissue [197]. Patients, especially postmenopausal women, who are taking SERMs while consuming alcohol are at a higher risk of recurrence [198]. The full extent of the impact of alcohol on ER-regulated and ER-independent mechanisms remains to be determined, including interactions between alcohol, estrogen, and SERMs used to treat hormone-dependent breast cancers. In this study, we investigated the effects of alcohol on growth factor and estrogen signaling, gene regulatory networks involved in clinical outcomes in breast cancer patients, the effects of alcohol on tamoxifen response in ER+ cell lines, as well as the functions of alcohol-regulated genes in breast cancer cell proliferation.

3.2 Materials and Methods

3.2.1 Cell Culture

Three standard human breast cancer cell lines were selected for use in these studies: MCF-7, T47D, and MDA-MB-231, (American Type Culture Collection, Rockville, MD, USA). MCF-7 cells were grown in high glucose Dulbecco's modified Eagle's medium buffered in HEPES (Invitrogen, Carlsbad, CA, USA). The media were supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA). T47D and MDA-MB-231 cells were grown in DMEM/F12 (Invitrogen) containing HEPES and glutamine. These cells were further supplemented with 10% FBS (Hyclone). Cells requiring estrogen-depeletion were washed in PBS and grown in DMEM or DMEM/F12 lacking phenol and supplemented with 10% charcoal/dextran filtered fetal bovine serum (Hyclone).

3.2.2 Cell Proliferation Assays, Cell Treatments, and Gene Knockdowns

Cells were treated with 10 nm 17β-estradiol (Sigma-Aldrich, St. Louis, MO, USA), 500 nm 4-hydroxytamoxifen (Tocris Bioscience, Bristol, UK), ethanol, or with DMSO as a vehicle. Cell proliferation was measured in one of two ways. Trypan blue exclusion assays were used to manually count cells using a hemocytometer. Otherwise, cell proliferation was measured using a standard MTS reagent, CellTiter96 Aqueous One Solution (Promega, Madison, WI, USA), according to the manufacture's standard protocol. For combination experiments in Figure 3.3, 7500 MCF-7 or T47D cells were seeded in a 96-well format, whereas 5000 MDA-MB-231 cells were similarly seeded for experimentation. Statistical analysis of these experiments was carried out using a standard two-tailed Student's t-test. All experiments were performed in triplicate. BRAF knockdown was accomplished by transfecting breast cancer cell lines with one of two targeting siRNAs following the standard manufacturer's protocol (Thermo Scientific Dharmacon, Lafayette, CO, USA). Scrambled siRNA from the same manufacturer were utilized as negative

controls. In these experiments, 5000 MCF-7 cells were seeded into a 96-well format for knockdown and subsequent MTS assays.

3.2.3 Western Blotting

Cells were starved of estrogen for 72 hours prior to indicated treatment conditions for 24 hours. Cells were then lysed in standard RIPA lysis buffer. Protein concentrations were determined with Qubit Protein Assay Kit (Invitrogen). 100 µg of protein was loaded into 10% polyacrylamide gels. After separation, the proteins were then applied to PVDF transfer membranes (Thermo Fisher Scientific, Rockford, IL, USA). After transfer, the membranes were blocked in TBST with 10% dissolved nonfat milk. After blocking, the membrane was probed with antibodies directed against pERK1/2 (Cell Signaling, Danver, MA, USA), ERK1/2 (Cell Signaling), BRAF (Santa Cruz), or GAPDH dissolved in 1% milk/TBST for 4 hrs to overnight. Membranes were washed of unbound or non-specific antibody and reprobed with horseradish peroxidase (HRP) specific secondary antibodies for 1 hr. Following a second wash, the film was exposed to ECL reagent (Thermo Fisher Scientific), to allow for their detection by blue autoradiographic film. Fold change quantification in protein levels was analyzed using the densitometric analysis package in ImageJ software (version 10.2) [199].

3.2.4 Illumina Bead Chip Arrays and Data Analysis

Total RNA from MC7-7 cells was isolated with RNeasy columns (Qiagen). 250 ng of RNA was converted to cRNA using the Illumina TotalPrep-96 RNA Amplification kit (Ambion, Carlsbad, CA,USA). Next, cRNA from the amplification kit was hybridized to the Illumina Whole-Genome Gene Expression Direct Hybridization Microarray (Illumina, San Diego, CA, USA). Duplicate probes were eliminated. R software packages *lumi* and *limma* were then used to identify differentially expressed genes in alcohol-treated MCF-7 cells. Overall intensity values obtained from this analysis were normalized. P values were then adjusted with the Benjamini-Hochberg correction used to account for false discovery in genome-wide analyses. Fold change values in

excess of 1.1 were then used to populate a list of responsive genes for data mining. Bioinformatic analysis of responsive genes was made in Pathway Studio (Ariadne Genomics, Rockville, MD). Fisher's exact test was used to determine statistically enriched pathways. The microarray data have been uploaded to the Gene Expression Omnibus repository and will be available to the public following publication (GSE66406).

3.2.5 Survival Analysis

Clinical microarray gene expression data generated from a cohort of breast cancer patients in Uppsala, Sweden were used to correlate responsive alcohol genes with disease parameters and outcomes [104]. Dendrograms were generated with Eisen Cluster and Treeview software. Survival analyses were generated using the survival plot functions (log-rank test) of Mathematica software. No consent or institutional review is required for this data as the analyses were based on previously published and publically available data.

3.2.6 Quantitative PCR

RNA from treated cells was extracted using the RNeasy Kit (Qiagen). Then, 0.5 µg of RNA was reverse transcribed using SuperScript III Reverse Transcriptase System (Invitrogen). Quantitative PCR was done on a 7500 Fast Real-Time PCR system (Applied Biosystems, Carlsbad, CA, USA) using Fast SYBR Green Master Mix (Applied Biosystems). Primer BLAST was then used to generate primers specific for these genes listed in Table 3.1. The ΔΔCt method was used to calculate fold changes between treatment conditions by normalizing to 36B4, a housekeeping gene (36B4 forward 5'-GTGTTCGACAATGGCAGCAT-3'; 36B4 reverse, 5'-GACACCCTCCAGGAAGCGA-3').

Table 3.1 Primers Used in Chapter 3		
Gene	**Forward Primer**	**Reverse Primer**
H19	5'-AGCCTCCACGACTCTGTTTC-3'	5'-TCCACAACTCCAACCAGTGC-3'
PPARg	5'-ATGAGTCTTCACCCGCTCCT-3'	5'-GGAAATGTTGGCAGTGGCTC-3'
WISP2	5'-CTGGGCTGATGGAAGATGGT-3'	5'-AGTGAGTTAGAGGAAAGGGGAC-3'
DHRS2	5'-GCAGAGGATTGGGGAGTCAG-3'	5'-TCAGAGCCGAGTGGAGTAGC-3'
SKP2	5'-CCCAGGAACTGCTCTCAAA-3'	5'-CTGCGGACAATCACAAAGT-3'
DHRS3	5'-AGCCACCTTGACACTTTTGAAC-3'	5'-TGGGTTTTGGAACGGGAGG-3'
BRAF	5'-TCTCACCAGTCCGTCTCCTT-3'	5'-TCCTCCATCACCACGAAATCC-3'
STIL	5'-CCAGCCACTTTCTGTATCC-3'	5'-GCACCCCCTGTTGGTC-3'
CYP26A1	5'-CCAGAAAGTGCGAGAAGAGC-3'	5'-TTCAGAGCAACCCGAAACCC-3'
TGFBI	5'-CCCTGAGAGACCTGCTGAAC-3'	5'-CGCCTTCCCGTTGATAGTGA-3'
ALDH1A3	5'-AGCAGCCGTGTTCACAAAAA-3'	5'-GTAGCAGTTGATCCAGACCGT-3'

3.3 Results

3.3.1 Ethanol Promotes Estrogen and Growth Factor Signaling Mechanisms in Breast Cancer Cell Lines.

To optimize the study of the effects of alcohol on breast cancer cell proliferation, several parameters were first established. MCF-7 breast cancer cells were primarily used in these studies because they are derived from the breast tumor subtype most commonly associated with alcohol consumption (ER+/PR+). They are also the most frequently used ER+ cell line and the most comprehensively studied. Cells were starved of estrogen (E2) in phenol-free medium supplemented with 10% charcoal filtered FBS for 72 hours prior to the specified treatments. Drugs and hormone compounds were dissolved in DMSO instead of ethanol to independently assess the effects of alcohol on breast cancer cell biology. After starvation and treatment, we performed trypan blue exclusion assays to evaluate whether alcohol is sufficient to drive ER+ breast cancer cell line proliferation in the absence of estrogen, which is a major target in these types of breast cancer. These results show that alcohol increased cell proliferation in MCF-7 cells only in the presence of estrogen (5.1A). Cells treated with ethanol in the absence of E2 did not proliferate more than cells treated without ethanol (p = .77, FC = 0.97). As a positive control, E2 significantly increased proliferation in ER+ MCF-7 cells over untreated cells (p < 0.001). Alcohol further promoted a 21% increase in cell proliferation in E2 treated cells (p = 0.006), demonstrating that conditions used in these studies were sufficient to evaluate breast cancer responses to alcohol. To establish the optimal working concentration of alcohol for use in functional studies, we performed a titration of alcohol concentrations in MCF-7 cells grown in cell medium containing estrogen, then subjected them to tetrazolium salt reduction assays (MTS), which measure mitochondrial metabolic rate and act secondarily as higher throughput reflections of cell number. In this assay, cells proliferated 24% more in response to 43.4 mmol/L ethanol and elicited the most potent response (Figure 3.1B) (p < 0.001 for 21.7, 43.4, and 65.1 mM ethanol treatments).

Cells treated to 86.8 mM ethanol were not statistically different from untreated cells, suggesting a suppressive effect on cell proliferation at this concentration. Furthermore, 43.4 mM ethanol was slightly more potent than 21.7 mM ethanol at increasing estrogen-dependent cell proliferation. This concentration was used for the remaining cell proliferation experiments. A blood alcohol content as low as 17.4 mM (~0.08%) begins to impair normal behaviors, and is considered a binge drinking episode [200]. Furthermore, the alcohol concentrations used in the cell proliferation experiments are higher than the amount attained in average binge episodes, but are lower than the achievable blood alcohol concentrations observed in alcohol patients [201].

Critical signals for estrogen-dependent cell proliferation are ERK1/2 phosphorylation, which is mediated though increased ER target gene transcription in response to estrogen, resulting in amplified HRG/HER2 signaling, and therefore increased growth [202]. To test whether alcohol modulates these signaling mechanisms, we carried out western blot experiments on combination estrogen and alcohol treated MCF-7 cells. These experiments showed that alcohol increased ERK1/2 phosphorylation (Figure 3.1C). Furthermore, pERK was increased 1.49 fold (p = 0.01), whereas the pERK of E2 treated cells was increased 2.3 fold (p = 0.001). Combination E2 and ethanol treatments increased pERK phosphorylation 2.8 fold relative to DMSO (p = 0.001). Alcohol promoted ERK phosphorylation in MCF-7 cells independent of estrogen treatment, but is still required for increased cell proliferation, suggesting estrogen-dependent and –independent mechanisms of alcohol activity in breast cancer cell lines. Despite an effect by alcohol on ERK phosphorylation levels, these experimental results demonstrated that alcohol is not sufficient to promote cell proliferation in the absence of estrogen.

Our interests in the effects of alcohol on estrogen signaling are based partially on previously published studies, which have shown that alcohol regulates estrogen receptor expression and transcriptional activity [189,203]. To confirm these results, MCF-7 cells were treated with alcohol and/or E2 and subjected to gene expression analysis. TFF1/pS2 and GREB1 are two well-known estrogen responsive genes (Figure 3.1D). GREB1 expression was amplified 15.09 fold in E2 and ethanol treated cells over DMSO. However, GREB1 was upregulated 11.46

fold in cells treated with E2 alone. This difference was statistically significant (p = 0.05). Expression levels of the TFF1/pS2 mRNA transcript, however, was not statistically different between E2 and E2/EtOH treated samples, suggesting that alcohol does not amplify the expression of estrogen responsive genes in a universal fashion.

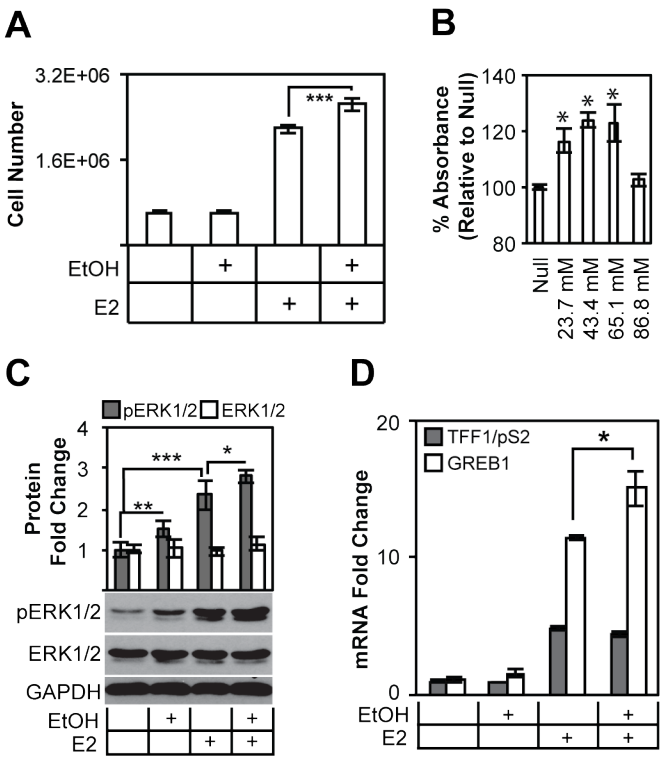


Figure 3.1. Alcohol increases cell proliferation in an estrogen-dependent manner, promotes the activation of ERK1/2, as well as known ER target genes.
(A), Trypan blue exclusion assays demonstrate that estrogen potentiates cell proliferation increases by alcohol. DMSO treated cells are not statistically different from DMSO and alcohol cotreatment. (B), MTS assays measure statistically significant increases in metabolic rate at 21.7, 43.4 and 65.1 mmol/L ethanol concentrations. Treatment with 86.8 mmol/L EtOH did not result in an increase in cell proliferation. (C) Alcohol promotes the phosphorylation of ERK1/2, a key effector of growth factor signaling and of G1-S progression, regardless of estrogen treatment. Quantification comprises data of experiments in triplicate. (D) The effect of alcohol was tested on ER responsive genes TFF1/pS2 and GREB1 in MCF-7 cells. Only GREB1 responds to alcohol treatment, suggesting a possible overlap between cellular estrogen signaling and alcohol response.

3.3.2 Alcohol Treatment Regulates Genes Involved in Key Cellular Processes that are Associated with Patient Survival and Response to Endocrine Therapy.

To better characterize potential mechanisms of alcohol action in breast cancer cells, ethanol-treated MCF-7 cells starved of estrogen were subjected to a genome-wide microarray analysis. Differentially expressed genes were defined by fold change cutoffs and false discovery corrected p-values. Overall, 898 genes were upregulated, and 654 genes were down-regulated by ethanol (Figure 3.2A). A small portion of these genes overlapped with known ER target genes [127]. Genes that were regulated by alcohol treatment independent of a known ER-binding site are termed “alcohol specific genes” in this analysis. 77 up-regulated ethanol responsive genes overlapped with the 904 previously identified ER target genes, whereas 37 down-regulated ethanol responsive genes overlapped with known ER target genes (Figure 3.2A). Gene ontology analysis showed that alcohol responsive genes regulated a wide variety of molecular pathways. Up-regulated alcohol-specific genes governed cell cycle and apoptotic genes. Furthermore, the down-regulated alcohol-specific genes are also involved in apoptosis, vesicle-mediated transport, and response to oxidative stress (Table 3.2). The limited number of genes that overlapped with estrogen signaling (ER target genes) were involved in cell migration, cell adhesion, and skin development (Table 3.3). Such a limited number of known estrogen responsive genes regulated by alcohol treatment suggested that alcohol was unlikely to independently regulate estrogen signaling or cell proliferation through ER. However, these results provide early leads into potential ER-independent mechanisms that are regulated by alcohol.

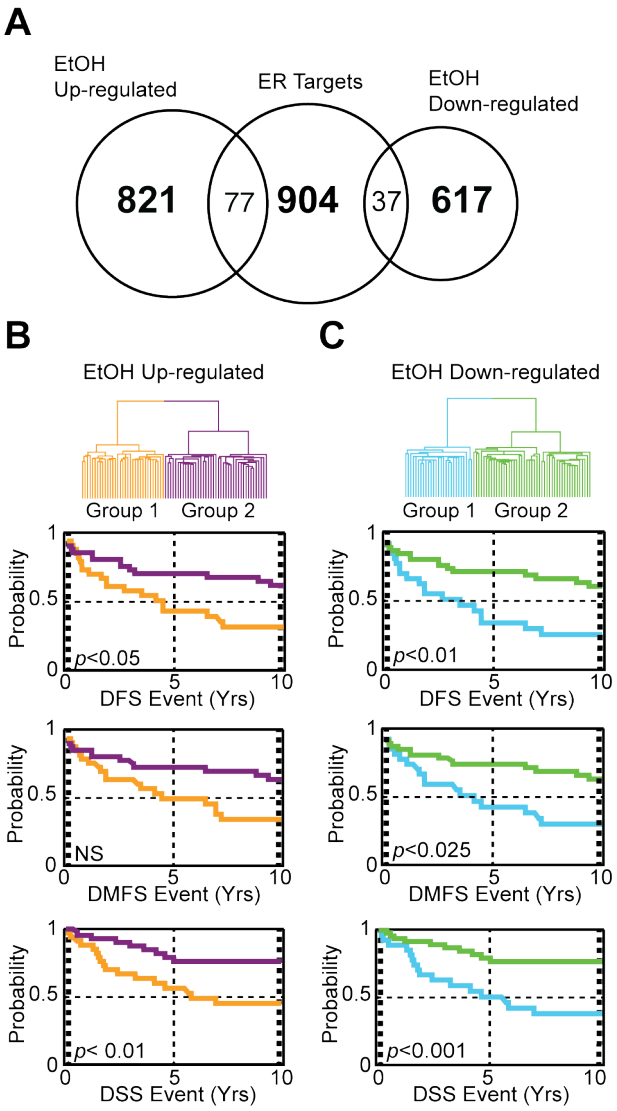


Figure 3.2. Gene networks regulated by alcohol treatment in MCF-7 cells are strongly correlated with breast cancer disease parameters.
(A) Representative Venn diagram demonstrating the number of up-regulated and down-regulated genes, as well as the overlap of ethanol responsive genes with ER target genes. (B) Up-regulated and (C) down-regulated alcohol responsive genes were analyzed for expression in a patient microarray (Upsalla database). Patients were then clustered into two groups based on their gene expression profiles. Parameters were correlated for DFS (disease-free survival), DMFS (mestastasis-free survival), and DSS (disease-specific survival). Survival plots of subdivided patient groups show that both up-regulated and down-regulated alcohol responsive genes are associated with clinical parameters and disease progression. (Patient dendrograms correspond to survival plots based on color).

Table 3.2. GO categories enriched in alcohol-specific responsive genes.

GO Category	Study/Category
Up-regulated Genes	
cell cycle	54/604
apoptosis	62/778
chromatin modification	32/262
protein ubiquitination	27/220
protein transport	49/602
protein phosphorylation	55/743
cell proliferation	38/429
interspecies interaction between organisms	32/325
RNA splicing	31/323
protein dephosphorylation	21/166
response to DNA damage stimulus	30/309
Down-regulated Genes	
oxidation-reduction process	59/840
transcription, DNA-dependent	113/2265
apoptosis	51/778
response to oxidative stress	19/150
multicellular organismal development	66/1146
vesicle-mediated transport	24/244
tRNA processing	14/89
viral reproduction	30/362
carbohydrate metabolic process	30/369
regulation of apoptosis	24/263

Table 3.3. GO categories enriched in alcohol-responsive ER target genes.

GO Category	Study/Category
Up-regulated Genes	
skin development	4/54
axon guidance	6/328
epithelial cell maturation	2/9
cell migration	4/141
response to chemical stimulus	3/65
mRNA polyadenylation	2/18
ectoderm development	2/20
transport	11/1812
epithelial cell maturation in salivary gland development	1/1
epithelial cell fate commitment	1/1
serotonin secretion, neurotransmission	1/1
Down-regulated Genes	
regulation of cell shape	2/76
lipopolysaccharide transport	1/2
detection of lipopolysaccharide	1/3
negative regulation of microtubule polymerization	1/4
recognition of apoptotic cell	1/4
cell adhesion	3/686
tyrosine catabolic process	1/5
cholesterol import	1/5
regulation of phagocytosis	1/6
positive regulation of cholesterol storage	1/6

To determine the potential clinical significance of alcohol responsive genes in breast cancer patients, we examined their expression profiles of alcohol responsive genes in a microarray dataset of breast cancers. This dataset contains expression data from a cohort of patients with corresponding morbidity and mortality data. Only patients with ER+ breast cancers being managed by endocrine therapy were included in this analysis, due to the clinical and pathological parameters that are associated with alcohol consumption. Hierarchical clustering of these patients was performed based on the expression profiles of alcohol up-regulated and down-regulated genes. Patients were then placed into one of two patient groups based on the hierarchical clustering patterns. The two groups were subsequently analyzed for disease-free survival (recurrence; DFS), distant metastasis-free survival (metastasis; DMFS), and disease specific survival outcomes (death; DSS). The up-regulated gene subset was associated with recurrence ($p < 0.05$) and death ($p < 0.01$) (Figure 3.2B), whereas the down-regulated subset clustered patients groups with very different recurrence, metastasis, and death outcomes ($p <$ 0.01, 0.025, and 0.001 respectively) (Figure 3.2C). Based on these survival analyses, it appears that alcohol responsive genes may serve as prognostic markers for patient response to endocrine therapy.

3.3.3 Alcohol Blocks Tamoxifen in ER+ Breast Cancer Cell Lines.

Due to the potential association of alcohol with response to endocrine therapy, we tested the hypothesis that alcohol may directly antagonize tamoxifen activity in breast cancer cells. In these experiments, we utilized MTS assays to measure metabolic rate in two standard ER+ cell lines (MCF-7 and T47D) and one ER- negative cell line (MDA-MB-231). As expected, the ER+ cell lines proliferated in response to E2 (80% in MCF-7 cells and 32% in T47D cells when compared to vehicle; $p < 0.001$ in both MCF-7 and T47D cells, Figure 3.3A-B). Furthermore, alcohol increased proliferation an additional 38% in MCF-7 cells and 23% in T47D cells over E2 alone ($p \leq 0.001$ in both cell lines). As a control, tamoxifen treatment suppressed E2 induction of cell

proliferation in both ER+ cell lines. To determine the association between alcohol and response to tamoxifen, ethanol co-treatment with E2 and tamoxifen increased cell proliferation 24.8% and 13.8% in MCF-7 and T47D cells respectively over E2 and tamoxifen treated cells ($p < 0.01$ in both cell lines). MDA-MB-231 cells did not respond to alcohol or ER ligands (Figure 3.3C). These data provide a direct link between alcohol responsive genes and previously published epidemiological data, in that expanded cell proliferation provided by estrogen and other factors is often a risk factor for the development of breast cancer [204]. These data also provide the mechanistic basis for the association between alcohol responsive genes and patient response to endocrine therapy.

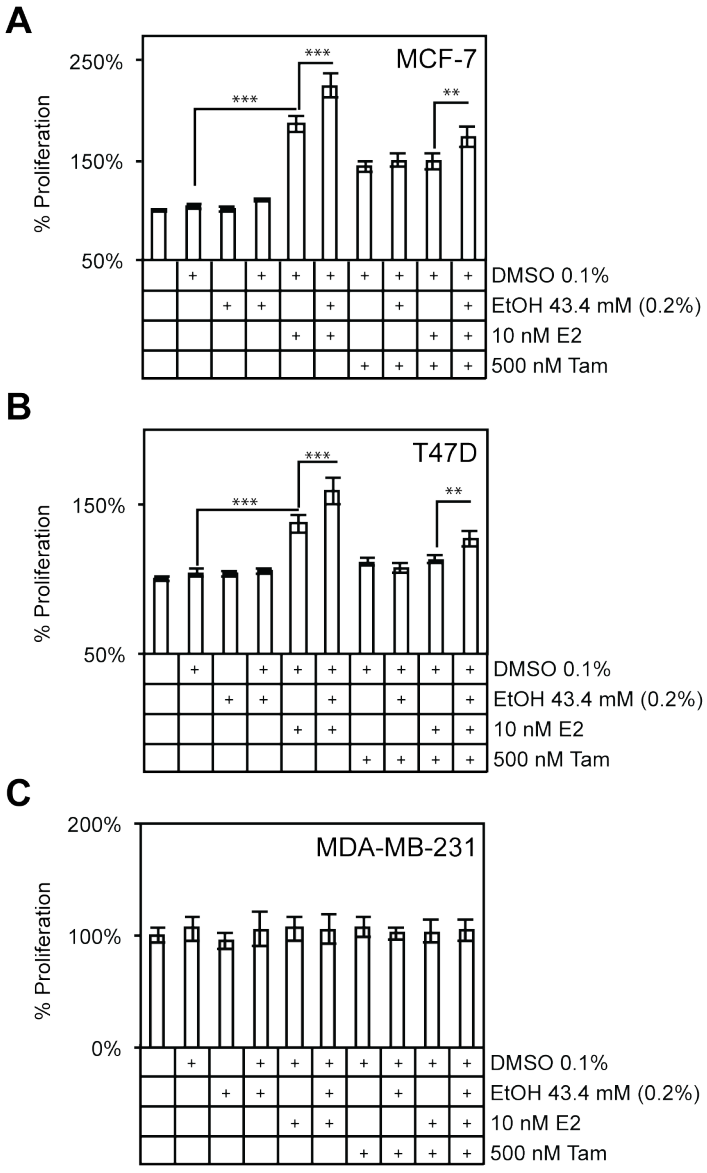


Figure 3.3. Alcohol enhances estrogen-dependent increases in cell proliferation and blocks tamoxifen attenuation of cell proliferation in MCF-7 and T47D cells.
(A), MTS assays demonstrate that alcohol is able to increase measures of metabolic rate in estrogen-treated MCF7 cells. Alcohol also largely blocks a dose of tamoxifen after 72 hours of treatment, suggesting a role for alcohol in breast cancer insensitivity to SERMS. (B) Similar results were observed in another ER+ cell line, T47D. (C) MDA-MB-231 cells do not respond to estrogen, tamoxifen, or ethanol.

3.3.4 BRAF is a Novel Ethanol Responsive Gene that Promotes Breast Cancer Cell Proliferation.

Previous analyses of ethanol responsive genes demonstrated a strong link between alcohol-responsive genes and clinical outcomes, but involved the clustering of patients based on a large number of alcohol-responsive genes (Figure 3.2). To ascertain the contributions of individual alcohol responsive genes to the phenotypes observed earlier, alcohol-responsive genes were analyzed for their differential expression based on clinical outcomes in ER+ breast cancers treated with endocrine therapy. Single genes with statistically significant differing expression levels were identified in patients who experienced recurrence (DFS), metastasis (DMFS), or death (DSS) (Table 3.4). Several, alcohol-responsive genes were identified in the microarray analysis and are involved in regulating cell proliferation (BRAF, SKP2, PPARG). These and other genes involved in the metabolism of alcohol were validated by qPCR (Figure 3.4A). The top responsive gene, BRAF, a downstream effector of growth factor signaling and a proto-oncogene, was induced 2.00 fold over untreated cells ($p < 0.05$) at the transcript level (Figure 3.4A). Ethanol promoted a 3.15 fold increase in BRAF protein levels in MCF-7 cells ($p = 0.008$). Treatment with E2 increases BRAF levels 3.15 fold ($p = 0.001$), whereas E2 and EtOH treatment increased BRAF levels 4.26 fold ($p = .004$). BRAF levels for MCF-7 cells treated with E2 and alcohol were not always increased over E2 treatment alone, but a distinct trend was present ($p = 0.18$) (Figure 3.4B). Taken together, these data show that BRAF is a novel alcohol and estrogen responsive gene, which is overexpressed in breast cancer patients with poorer DSS parameters.

Table 3.4. Alcohol-responsive genes differentially expressed between outcome groups.

Gene	DFS	DMFS	DSS
Up-regulated Genes			
STIL	**0.002**	**0.004**	**0.001**
ASCL1	**0.019**	**0.035**	**0.003**
TULP4	0.244	**0.008**	**0.005**
RIF1	0.294	**0.02**	**0.005**
MIER3	0.002	**0.007**	**0.007**
BRAF	0.176	0.054	**0.014**
ID2	0.225	0.263	**0.016**
SKP2	0.505	0.081	**0.022**
TP53INP1	0.081	0.059	**0.022**
PHIP	0.594	0.65	**0.049**
Down-regulated Genes			
WISP2	**0.001**	**0.001**	**0.001**
DIO2	**0.013**	**0.007**	**0.002**
H19	**0.005**	**0.005**	**0.011**
PPARG	0.132	**0.048**	**0.014**
VEGFB	**0.012**	**0.023**	**0.020**
RBPMS	0.121	0.141	**0.025**
DICER1	**0.008**	**0.008**	**0.026**
DHRS2	0.676	0.633	**0.039**
ITGB5	**0.002**	**0.01**	**0.048**
VGF	**0.005**	**0.014**	**0.048**

DFS: Disease-free Survial (Recurrence)

DMFS: Distant Metastasis-free Survial (Metastasis)

DSS: Disease-specific Survial (Death)

Due to its known roles in cell proliferation and oncogenesis, we examined the effect of BRAF on alcohol response in MCF-7 cells. First, BRAF siRNA knock-down suppressed BRAF transcript and protein levels (Figure 3.4C). We then performed knock-downs of BRAF and determined their effects on cell proliferation using MTS assays. Knock-down of BRAF attenuated basal proliferation rates, as well as estrogen-dependent proliferation in MCF-7 cells. Furthermore, knock-down of BRAF was able to partially attenuate alcohol response, especially with construct 2 (Figure 3.4C). Furthermore, BRAF regulated basal, as well as estrogen-dependent proliferation in MCF-7 cells. Lastly, Kaplan-Meier survival analysis of patients based on BRAF expression levels showed statistically significant DMFS and DSS outcomes in ER+ breast cancer patients (p = .02 and 0.03, respectively), where women with higher expression of the BRAF mRNA responded more poorly to endocrine therapy (Figure 3.5). These results identified BRAF as a novel alcohol responsive gene, whose expression is correlated with disease outcomes, and is involved in breast cancer cell proliferation.

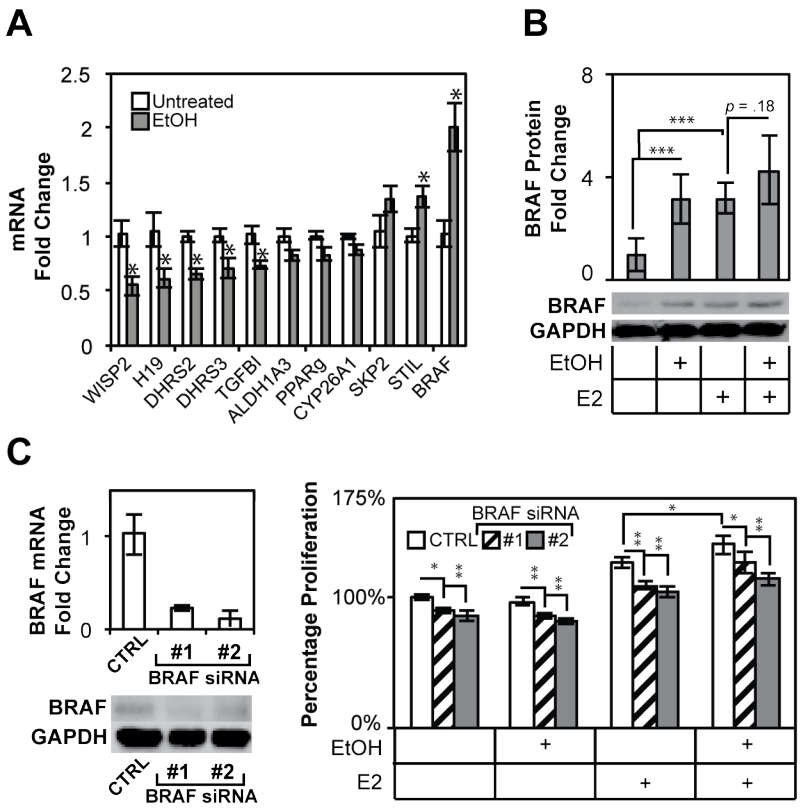


Figure 3.4. Alcohol regulates BRAF, an effector of growth factor signaling, and promotes estrogen-dependent and –independent growth.
(A), Microarray validation demonstrates subtle but highly reproducible effects on gene expression of down-regulated and up-regulated genes. (B) BRAF is up-regulated at the protein level by alcohol and estrogen treatment. (C) BRAF is targetable with siRNA knockdown for functional studies. MTS assays demonstrate the anti-proliferative effect of BRAF knockdown on MCF-7 cells, suggesting that BRAF promotes basal cell proliferation in the absence of estradiol, increases estrogen-dependent growth, and potentiates some of the cell's response to ethanol.

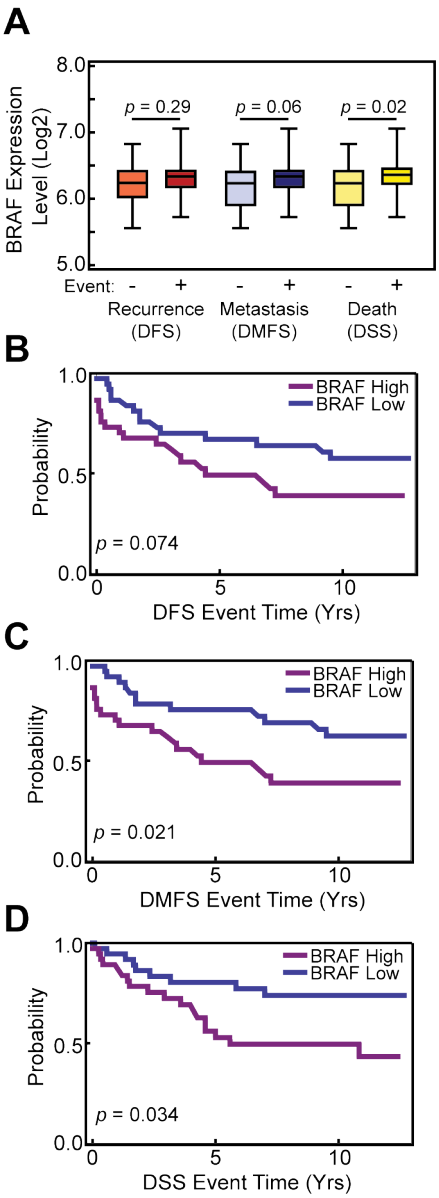


Figure 3.5. High BRAF expression levels correlated ER+ endocrine treated patients with poor prognosis and response to therapy.
(A) BRAF is expressed at higher levels in patients who experience metastasis or do not survive breast cancer. BRAF is marginally higher in patients who experience a recurrence, but this is not statistically significant. (B) BRAF expression levels separate patients into different DFS groups, albeit not statistically different (p = 0.074). Statistically different (C) DMFS and (D) DSS groups are observed in ER+ endocrine treated patients based on high expression levels of BRAF.

3.4 Discussion

The aim of this study was to identify molecular pathways that contribute to alcohol response in ER+ breast cancer cells. We first established that alcohol increased estrogen (E2)-dependent cell proliferation but it did not promote proliferation in estrogen-starved cells (Figure 3.1A), demonstrating that estrogen potentiated alcohol-induced cell proliferation. This contrasts with other studies, which were not able to identify the link between alcohol and estrogen in cell proliferation assays [203], due to the lack of experiments performed on cells grown in estrogen-depleted medium containing growth factors. Furthermore, we also identified the optimal concentration of alcohol for evaluating proliferative responses in breast cancer cells while maintaining physiologically attainable levels of alcohol. The most robust proliferative response was observed in MCF-7 cells treated to 43.4 mmol/L alcohol (Figure 3.1B). To address concerns of potential cytotoxic effects of alcohol, previously published studies determined that cytotoxicity occurs at very high levels of alcohol treatment (> 425 mmol/L), concentrations which were not evaluated in our study [194]. However, we found that the alcohol-dependent proliferative concentration window (between 21.7 mmol/L and ~65.1 mmol/L alcohol) was much lower than the cytotoxic dose of alcohol (>425 mmol/L alcohol). These studies clarified the optimal parameters for studying ethanol response, which was estrogen-dependent and fell well below cytotoxic thresholds observed in other studies.

Estrogen signaling regulates and is highly integrated with growth factor signaling networks. We determined that alcohol promoted a known key regulator of estrogen-induced cell proliferation, the phosphorylation of ERK1/2, independent of estrogen (Figure 3.1C). Phosphorylated ERK1/2 are required for G1-S transition, and are thought to grant permissions for early events in G1 by up-regulating pyrimidine synthesis, regulating protein translation, or activating transcription factors involved in subsequent cell cycle processes [205-208]. It appears from these results that alcohol promoted both estrogen- and alcohol-specific responses, as increased pERK1/2 did not result in increased proliferation in the absence of estrogen. Potential mechanisms of ERK1/2 regulation have been proposed in other studies. Increased ERK signaling

could be due to the inactivation of phosphatases by reactive oxygen species (ROS) generated from alcohol detoxification, allowing for the accumulation of activating phosphorylation marks on growth factor receptors [209-211]. An alternative mechanism of the effects of alcohol on growth factor signaling pathways is that alcohol generated ROS lead to the inappropriate activation of matrix metalloproteinases, which are known to stimulate the activity of growth factor signaling ligands [193,211]. Regardless, these results form an important link between the two critical pathways in breast cancer, growth factor signaling and estrogen signaling, which are both regulated by alcohol.

Alcohol has been shown to up-regulate the expression of an estrogen-responsive luciferase reporter gene [189], an effect which was shown to require estrogen. However, the effect of alcohol on the expression of ER target genes on endogenous promoters has not been extensively explored. We showed that alcohol further increased GREB1 expression after estrogen treatment, suggesting that alcohol promotes hyper-activation of estrogen signaling in breast cancer cells (Figure 3.1D). TFF1/pS2, however, did not respond to alcohol treatment, possibly due to ER saturation of that promoter, negative feedback loops on transcription of the gene target, treatment time conditions, or was otherwise insensitive to the effected mechanisms of alcohol treatment. These findings contrasted with another study that described a TFF1/pS2 response to alcohol treatment, albeit the regulation was relatively subtle. We were unable to reproduce this effect in MCF-7 cells, possibly due to differences in experimental design [203].

From genome-wide microarray studies of alcohol treated cells, we first observed that a significant proportion of the genome responded to alcohol treatment (Figure 3.2A). Due to the depletion of estrogen in the cell culture medium, we were able to assess whether alcohol could transactivate ER target genes independent of estrogen. Surprisingly, given the published reports of the effects of alcohol on ER, relatively few estrogen responsive targets were found to overlap with alcohol responsive genes, suggesting that alcohol is not an estrogen mimetic. These data also suggest, especially in light of the estrogen-independent effect of alcohol on ERK1/2 phosphorylation, that alcohol-specific genes may potentially enhance estrogen dependent cell

proliferation. To test this hypothesis, alcohol responsive genes were further analyzed for statistically enriched gene ontology categories. Cell cycle genes (CCND2, RAD17, EP300) were up-regulated in MCF-7 cells treated with alcohol (Table 3.2).

Genes involved in protein phosphorylation (ROCK1/2, JAK2, SMAD5) and dephosphorylation (DUSP1/12, BCL2, PTP) were also regulated by alcohol treatment. As previously mentioned, cell cycle machinery is heavily dependent upon posttranslational modifications for correct regulation of growth factor signaling cascades, which could explain the enrichment of gene ontology categories involved in general protein phosphorylation. Genes involved in oxidative-reduction responses (P53, SOD1, HMOX1) and apoptotic genes (CASP2, BID, VIM) were enriched in the down-regulated alcohol specific gene subset. These data indicate that alcohol regulates a number of pathways that have known critical roles in breast carcinogenesis.

To ascertain the clinical significance of the alcohol-responsive genes, their expression profiles and association with disease outcomes were analyzed in a microarray dataset from tumors obtained from a cohort of patients who received endocrine therapy. Expression profiles of both up-regulated (Figure 3.2B) and down-regulated (Figure 3.2C) genes were strongly associated with metastasis (DMFS) and death (DSS). It is not clear from these data what roles these genes, as a whole, may play in breast carcinogenesis, disease progression, and response to SERMs, but their association with response to endocrine therapy suggests that alcohol treatment affects the expression of a large number of genes which, at the very least, are prognostic markers of therapeutic response and may function in key molecular pathways and mechanisms. At the molecular level, normal ER activity in breast cancer cells is antagonized by SERMs, which prevents estrogen-dependent cell proliferation [212,213]. Due to the differences in patient outcomes based on the gene expression profiles in patients of alcohol responsive genes, we suspected that alcohol might promote breast cancer cell proliferation even in the presence of tamoxifen. In agreement with this hypothesis, we determined that alcohol treatment attenuated tamoxifen suppression of cell proliferation in MCF-7 (Figure 3.3A) and T47D cell lines (Figure

3.3B). MDA-MB-231 cells did not respond to any of the treatment conditions, suggesting that ER and ER- associated factors mediate the effects of tamoxifen and alcohol. Several mechanisms of tamoxifen insensitivity have been previously identified. BRCA1 levels have been shown to be down-regulated by alcohol treatment [189]. Down-regulated BRCA1 levels lead to increased cell proliferation in the presence of tamoxifen by altering its interactions with transcriptional coregulators and alter the nature of ligand-bound ER and its downstream transcriptional responses [213]. Alternatively, amplified growth factor signaling can lead to increased cell proliferation in the presence of tamoxifen. In this study, we showed that growth factor signaling (pERK1/2) is activated in response to alcohol treatment, which has been shown in other studies to be up-regulated in tamoxifen resistant tumors (Figure 3.1C) [212]. These data together provide experimental evidence that alcohol can directly block the effects of tamoxifen and may lead to poor clinical outcomes and responses to therapy.

To further determine the mechanisms of action of alcohol in breast cancer biology, individual responsive genes were analyzed for differential expression based on clinical outcomes and response to endocrine therapy in ER+ breast cancer patients (Table 3.4). WISP2, for instance, is consistently down-regulated in ethanol treated cells, and has been shown to prevent migration in MCF-7 cells by up-regulating E-cadherin expression and down-regulating MMP9 activity [190]. The repressed gene dehydrogenase/reductase enzyme 2 (DHRS2) is expressed in MCF-7 cells, and is more highly expressed in luminal cells compared to basal cells, suggesting a link between higher expression of this protein and a less aggressive luminal phenotype [214]. *H19*, or maternally expressed *H19,* is a long non-coding RNA that has been shown to attenuate let-7 activity, a microRNA that regulates cell proliferation and apoptosis [215]. Deletions of the H19 mRNA have also been shown to lead to overgrowth in transgenic mouse models, possibly due to disrupted IGF-2 regulation [216]. Short-chain dehydrogenase/reductase 3 (DHRS3) has been identified as a p53 responsive gene, and functions to reduce all-trans-retinal to replenish bleached retinoids in the visual cycle [217]. DHRS3 is potently induced by retinoic acid, an antiproliferative vitamin-A derivative so alcohol may interact with vitamin-A associated pathways

in breast cancer cell lines [218]. Transforming growth factor β-induced (TGFBI) is a secreted protein and is also responsive to retinoic acid treatment in MCF-7 cells and has been shown to prevent both anchorage-independent growth in MCF-7 cells and the development of metastatic lesions in mouse xenograft models [42] [219]. The SCL/TAL1 (STIL) interrupting locus gene is required for cell-cycle progression, as well as for centriole biogenesis and function [220,221]. STIL attenuation prevents tumor growth in mouse colon cancer xenograft models [222]. The functional studies in this paper focused on BRAF, an effector of the growth factor signaling and upstream regulator of the mitogen-activated protein kinase/ERK cascade and a therapeutic target in other cancers such as melanoma [223,224]. The observed effects of up-regulated ERK1/2 phosphorylation in response to alcohol treatment suggest a role for BRAF in alcohol responsive signaling pathways and effects (Figure 3.1C). BRAF is a novel alcohol- and estrogen-responsive gene, and its transcript levels were negatively correlated with patient survival and response to endocrine therapy (Figure 3.5). These findings suggest that alcohol inappropriately promotes sustained expression of BRAF, even in the absence of estrogen, in women who consume alcohol and may thereby mimic or enhance the effects of estrogen in increasing breast cancer risks. These findings not only shed light on mechanistic actions of alcohol in breast cancer but also provide insights into the cross-talk between alcohol and known and novel oncogenic pathways in breast cancer in general

3.5 Conclusions and Future Directions

Our interests in the effects of alcohol on estrogen signaling are based partially on previously published studies, which have shown that alcohol regulates estrogen receptor expression and transcriptional activity [189,203]. In this study, we investigated the effects of alcohol on estrogen and growth factor signaling. We were able to demonstrate enhanced cell proliferation and increased markers of growth factor signaling in breast cancer cells. Future experiments should attempt to identify causal links between alcohol treatment and growth factor signaling, which would allow for a more complete atlas of signaling with regards to alcohol in breast cancer. We then identified gene networks regulated by alcohol that were involved in clinical outcomes in breast cancer patients, and determined the effects of alcohol on the response of ER+ cell lines to tamoxifen treatment. These characterizations are important, especially for breast cancer patients who are currently undergoing treatment for their breast cancer, or have had breast cancer and are in remission. We then characterized the effects of individual alcohol responsive genes on breast cancer cell biology. Future studies on these mechanisms will hopefully address several open questions with regards to alcohol response in breast cancer cells. First, a more complete analysis of the effects of alcohol on estrogen signaling is needed.

To better understand the effects of alcohol on estrogen response, a four-part microarray is required that adds an estrogen treatment to alcohol treated cells (as the original microarray study presented here was carried out on estrogen starved cells treated with alcohol). This would allow us to test the hypothesis that alcohol amplifies estrogen dependent transactivation. Due to the increase transcription of these genes, chromatin immunoprecipitation experiments can be carried out on the alcohol associate EREs, allowing for the identification of increased ER binding or stability.

Changes to metabolism of NR signaling compounds were suspected due to the repression of a subset of retinoic acid responsive genes in our microarray study. Very little is known about endogenous retinoids in breast cancer. However, Chen et al. carried out a study wherein radioactive retinol was incubated in the cell culture medium of breast cancer cell lines

[225]. The cells were then able to metabolize retinol into a variety of forms. Breast cancer cell lines do not follow the standard metabolic pathways observed in the eye and adipose tissue, which involves the conversion of retinol to retinoic acid. ER+ breast cancer cell lines convert retinol to 4-oxoretinol instead. Interestingly, 4-oxoretinol has been shown to suppress cell proliferation similar to retinoic acid treatment. With an endogenous RXR/RAR ligand in mind, we carried out HPLC experiments to determine whether alcohol altered intracellular retinoid levels. There was no difference in intracellular retinol levels due to alcohol treatment, but the HPLC instrument lacked the sensitivity to detect 4-oxoretinol in MCF-7 breast cancer cells. Detection of these compounds require incubation with radioactive retinol and subsequent analysis on the appropriate HPLC instrument.

Lastly, studies of the effects of alcohol in mouse models are severely lacking. Animal studies are necessary because they measure disease parameters that are deficient in a cell culture, such as the effects of other cell types (stromal cells) and secreted factors that serve ultimately to model disease progression in human cancer patients. Currently, only two studies have been carried out to determine the effect of alcohol on breast cancer progression in live animals. In both cases, lung metastasis was their experimental readout. One study showed a suppressive effect of alcohol on metastasis, which is in contradiction to the pro-carcinogenic role in our studies [195]. However, this study utilized an ER- cell line, which does not represent the standard pathological breast cancer subtype associated with alcohol consumption, as these as alcohol related cancers tend to be ER+/PR+. Another study used an ER+ cell line but carried out the experiment in male mice that lack estrogen, the driving force of proliferation in ER+ cell lines [196]. These results therefore do not address the effects of alcohol in a context that has been shown to potentiate its formation and development. Future studies on mouse models of breast cancer should also test the effects of alcohol on tamoxifen activity, which we have shown to be attenuated by alcohol treatment. These findings and future directions shed light on mechanistic actions of alcohol in breast cancer, and are suggestive of extensive cross-talk between several known oncogenic pathways in breast cancer.

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