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Exploring the Regulatory Mechanism of the Notch Ligand Receptor Jagged1 Via the Aryl Hydrocarbon Receptor in Breast Cancer

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EXPLORING THE REGULATORY MECHANISM OF THE NOTCH LIGAND RECEPTOR JAGGED1 VIA THE ARYL HYDROCARBON RECEPTOR IN BREAST CANCER

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Approved by
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APPROVAL OF DISSERTATION

We, the faculty supervising the work of Sean Alan Piwarski, affirm that the dissertation, *Exploring the Regulatory Mechanism of the Notch Ligand Receptor JAGGED1 via the Aryl Hydrocarbon Receptor in Breast Cancer*, meets the high academic standards for original scholarship and creative work established by the Biomedical Sciences program and the Graduate College of Marshall University. This work also conforms to the editorial standards of our discipline and the Graduate College of Marshall University. With our signatures, we approve the manuscript for publication.

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DEDICATION

To my mom and dad,

This dissertation is a product of your hard work and sacrifice as amazing parents. I could never have accomplished something of this magnitude without your love, support, and constant encouragement. Thank you for teaching me that anything worth having must be earned and that what I am willing to accomplish is determined by what I am willing to sacrifice. Having you two in my life gave me the courage to acknowledge my self-worth and the tenacity to never quit, even in the most improbable situations.

I love you and thank you for everything,

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ABSTRACT

The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor that binds pollutants, therapeutic drugs and endogenous ligands. AHR is of particular interest in cancer and has been shown to play roles in both tumor progression and tumor suppression. As a result, it has received growing attention as a possible chemotherapeutic target. AHR is expressed in all breast cancer subtypes and can promote or inhibit breast cancer depending on the ligand it binds. The Notch signaling pathway is a highly conserved evolutionary pathway that plays extremely vital roles during development by regulating cell fate and differentiation. Notch signaling has increasingly attracted attention as a therapeutic target for cancer treatments and ligand-induced Notch activation has been reported to promote the progression of several cancers including breast cancer. Jagged 1 (JAG1) is a Notch receptor ligand that is overexpressed in all breast cancer subtypes, including triple negative breast cancer (TNBC). JAG1 promotes various vital functions of cancer biology including cancer stem cell maintenance, drug-resistance, epithelial-mesenchymal transition (EMT), and metastasis. The regulation of JAG1 by AHR in breast cancer cells via two AHR ligands, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and 2-(1'H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methylester (ITE), were investigated for this dissertation. TCDD is the prototype AHR ligand, and ITE is a non-toxic endogenous AHR ligand with anti-cancer activity. Our laboratory has discovered that ligand-activated AHR inhibits the expression and activity of the JAG1-Notch pathway in human breast cancer cells, which in turn decreases breast cancer cell invasion. By conducting ribonucleic acid (RNA)-sequencing and analyzing the data via Ingenuity Pathway Analysis (IPA), we identified a significant association between TCDD-regulated genes (TRGs) and cell movement. We found that silencing AHR expression by short-interfering RNA (siRNA) or antagonizing its activity with the AHR

antagonist CH-223191 in breast cancer cells restored JAG1 expression, which established that ligand-activated AHR is an inhibitor of the JAG1-Notch pathway in breast cancer cells. AHR was also found to be necessary for suppressing the expression of the EMT regulator Snail, a crucial protein required for promoting cancer metastasis. Finally, we have shown that other non-toxic AHR agonists such as tranilast also decreases JAG1 expression in TNBC cells. Collectively, my work is the first to show that ITE is a tumor-suppressing AHR ligand in breast cancer cells in part because it reduces JAG1 expression. The findings presented in this dissertation suggest targeting the JAG1-Notch pathway with non-toxic AHR ligands could be a new mechanism to suppress the invasive activity of TNBC, which is a breast cancer subtype for which there are no targeting therapies.

CHAPTER 1

OVERVIEW OF CURRENT KNOWLEDGE IN BREAST CANCER

1.1. Survival Rates and Subtypes

There are more than 3.5 million women with breast cancer in the United States (American Cancer Society, 2016). Breast cancer is a heterogeneous disease that has been clinically organized into four subtypes (Figure 1) based on certain molecular markers: 1) Luminal A (Estrogen Receptor (ER)+/Progesterone Receptor (PR)+/Human Epidermal Growth Factor Receptor 2 (HER2)-) makes up ~40-50% of breast cancers, 2) Luminal B (ER+/PR+/HER2+) makes up ~15-20% of breast cancers, 3) HER2-enriched (ER-/PR-/HER2+) makes up ~8-10% of breast cancers and 4) triple negative breast cancer (TNBC) (ER-/PR-/HER2-) makes up ~15-20% of breast cancers (Keegan, DeRouen, Press, Kurian, & Clarke, 2012). Women with Luminal A tumors have the best 10-year survival rates (70%), compared with women with Luminal B (54%), HER2-enriched (48%), and TNBC (53%) tumors (Kennecke, et al., 2010). Women with Luminal A and B, and HER2-enriched subtypes can be treated with anti-estrogen or HER2 targeting therapy, respectively. However, TNBC lacks molecular targets for specific cancer targeting. Currently, women with TNBC are treated with chemotherapy and endure the associated toxicities of these non-specific drugs.

Recent studies have identified a small subpopulation of cells within breast tumors that exhibit stem-cell like characteristics that confer these cells with heightened tumorigenic activity, drug resistance (Gottesman, Fojo, & Bates, 2002; Ma & Allan, 2011), tumor recurrence (Yi, Kabha, Papadopoulos, & Wagner, 2014; Bosukonda & Carlson, 2017) and metastatic activity (Xie, et al., 2012; Uchino, et al., 2010). This small subpopulation of cells has been termed cancer stem cells (CSCs) and they are driven by signaling pathways that are triggered by

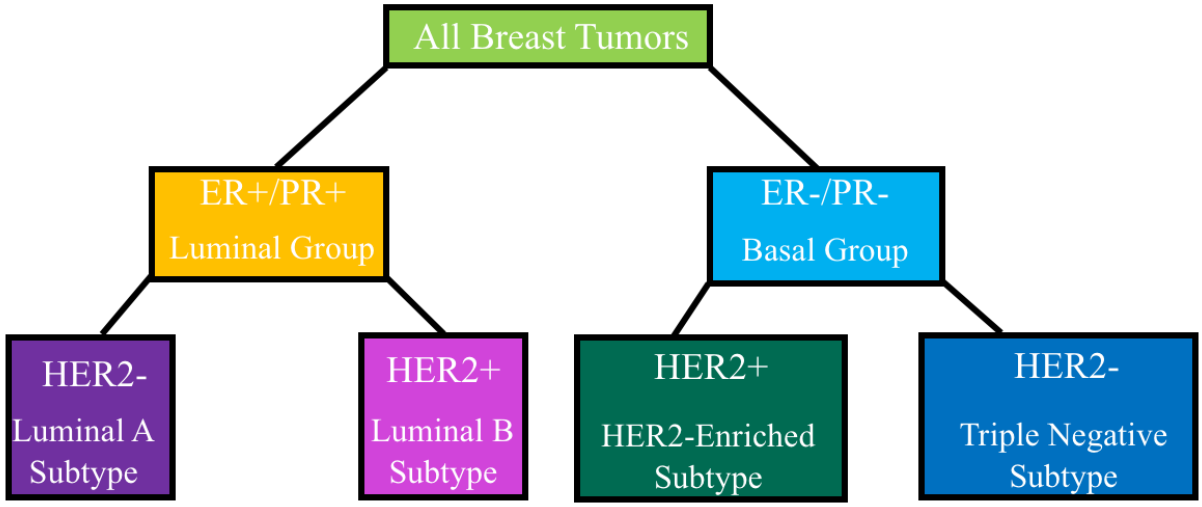


Figure 1. Subtypes of Breast Cancer Tumors
Each subtype has a different prognosis and treatment response. For ER+/PR+ group, because ER is a therapeutic target, the Luminal A and Luminal B subtypes are amenable to hormone therapy. Similarly, the HER2 groups are potential candidates for trastuzumab therapy. For the ER-/PR- group, in the current absence of expression of a recognized therapeutic target, basal tumors are difficult to treat, more biologically aggressive and often have a poor prognosis. This is because the basal phenotype is characterized by the lack of expression of ER, PR and HER2 and is sometimes referred to as triple negative breast cancer. TNBC = Triple Negative Breast Cancer, ER = Estrogen Receptor, PR = Progesterone Receptor, HER2 = Human Epidermal Growth Factor Receptor 2.

extracellular signals, mutations, and epigenetic control (Hartwig, et al., 2014). In this section, the subtypes of breast cancer, breast cancer therapies and breast cancer stem cells (BCSCs) will be discussed.

1.2. Breast Cancer Subtype Characteristics and Current Treatment Strategies

1.2.1. Luminal A and B Subtype

The Luminal A and B subtypes are highly associated with proliferation/cell cycle-related pathways and luminal/hormone-regulated cell processes (Prat, et al., 2015). The ER is expressed in the Luminal A and Luminal B breast cancer subtypes. The five and ten-year survival rates for Luminal B breast cancer are worse than for Luminal A breast cancer, irrespective of therapy. Compared with Luminal A breast cancer, Luminal B breast cancer exhibits higher expression of proliferation/cell cycle-related genes and proteins as well as lower expression of several genes that link with luminal breast cells, including the PR (Prat, et al., 2013). The analysis of mutation rates has demonstrated that Luminal A breast cancer exhibits fewer mutations than Luminal B breast cancer. The Luminal A subtype has a lower percentage of gene amplification events such as lower rates of cyclin D1 (CCND1) gene amplification, fewer tumor protein 53 (TP53) mutations (12% vs. 29%), and a higher rate of phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha (PIK3CA) (45% vs. 29%) and mitogen-activated protein kinase kinase kinase 1 (MAP3K1) mutations (13% versus 5%) compared to Luminal B tumors (Cancer Genome Atlas Network, 2012). The differences in these particular molecular markers (i.e., PR, CCND1, TP53, PIK3CA and MAP3K1) are used to distinguish Luminal A breast cancer from Luminal B breast cancer.

Luminal A and Luminal B breast cancers are responsive to chemotherapy in a neoadjuvant setting in which treatment is given as a first step to shrink a tumor before surgery.

Although surgery is the frontline therapy, Luminal A and Luminal B breast cancers are also treated with chemotherapy, radiation therapy, and hormone therapy. A clinical study was conducted on 208 women with Luminal A or Luminal B breast cancer that were treated with anthracycline/taxane-based chemotherapy. According to the pathologic complete response (pCR) data, which is the absence of residual invasive disease in the breast and in the auxiliary lymph nodes at the completion of the neoadjuvant treatment, the pCR rates in patients with Luminal A and Luminal B subtypes were 3% and 16%, respectively (Prat, Parker, & Fan, 2012). The results from a study comparing Luminal A to Luminal B breast cancer identified that women with low-proliferating Luminal A breast tumors were more responsive to paclitaxel compared with women that had high-proliferating Luminal B tumors (Martín, et al., 2013).

The use of drugs that specifically target estrogen signaling in Luminal A or Luminal B breast cancer has been in clinical practice for nearly fifty years (Lumachi, Santeufemia, & Basso, 2015). The estrogen targeting drugs target several different aspects of estrogen signaling in breast cancer (Figure 2). For instance, aromatase converts androgen into estrogen (Brueggemeier, Hackett, & Diaz-Cruz, 2005). The aromatase inhibitors (AIs), anastrozel and letrozol, bind to aromatase and block its activity, which reduces estrogen production. AIs are approved for the treatment of ER positive breast cancer in postmenopausal, but not premenopausal, women. Aromatase inhibition is ineffective in premenopausal women with functionally active ovaries, because reducing estrogen levels in these women with AIs induces a compensatory increase in the secretion of luteinizing hormone (LH) and follicle stimulating hormone (FSH), which in turn increases aromatase activity leading to increases in estrogen levels (Ma, Reinert, Chmielewska, & Ellis, 2015). The selective estrogen receptor modulators (SERMs) include tamoxifen, raloxifen and toremifine. The SERMs bind to the ER and have tissue-specific

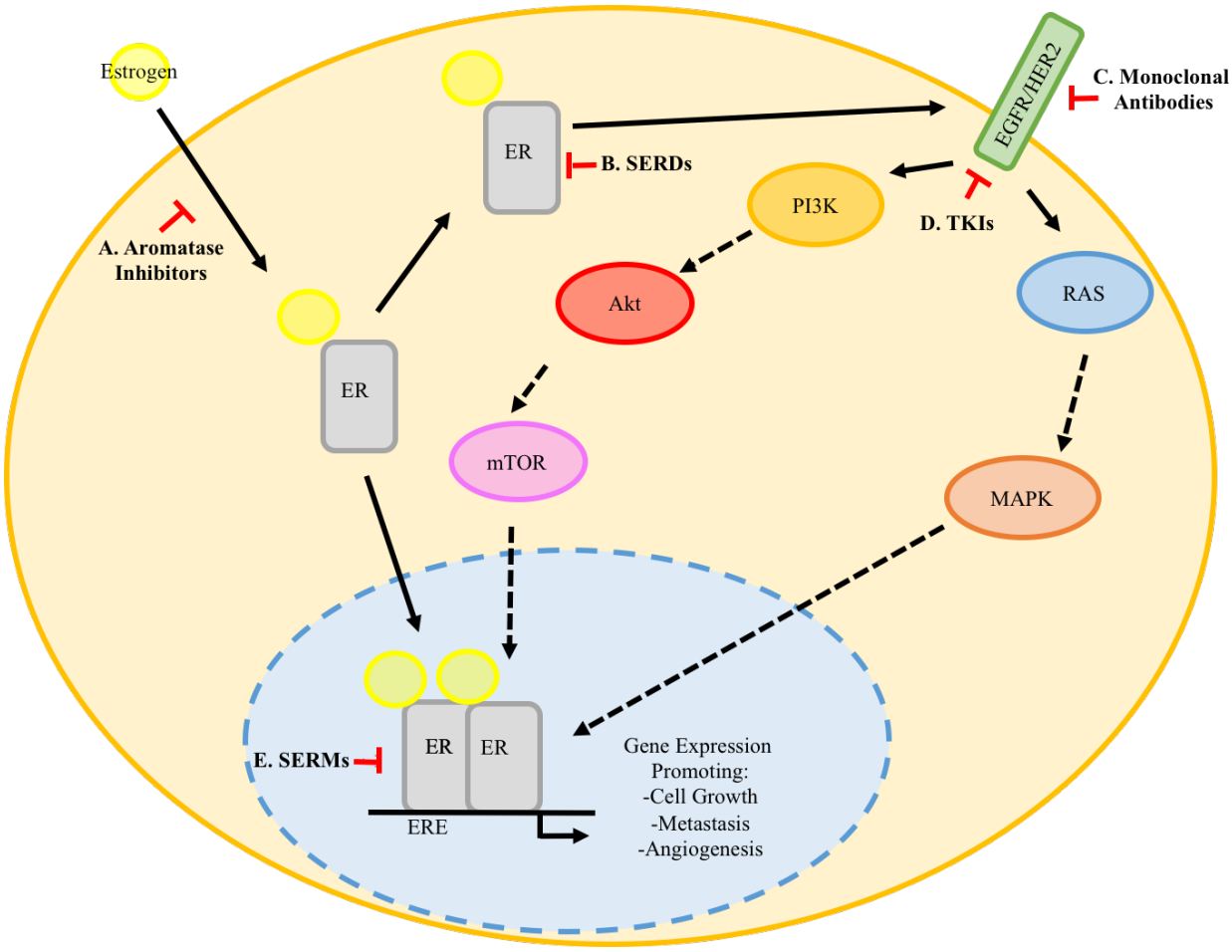


Figure 2. Current Strategies for Endocrine Therapies in Breast Cancer
Estrogen activates nuclear ER (genomic pathway) and ER in or near the membrane (non-genomic pathway). Membrane associated ER binds to growth factor signaling components such as PI3K. Estrogen then activates growth factor signaling, activating key molecules such as Akt or RAS, and downstream molecules such as mTOR and MAPK, which promote cell proliferation and survival. (A) AIs assist in decreasing the synthesis of estrogen as an approach for reducing growth-stimulatory effects. (B) SERDs function as high affinity competitive antagonists and induce a conformational change that is incompatible with co-regulator interactions and target the receptor for proteasomal degradation. (C) Humanized monoclonal antibodies target different growth factor receptors that promote cancer growth by blocking their activation. (D) Small-molecule TKIs bind the intracellular domains of growth factor receptors to block activation of their downstream signaling pathways. (E) SERMs act as a competitive receptor inhibitor in the breast tissue by selectively blocking ER signaling and inhibiting the proliferation of tumor cells. ER = Estrogen Receptor, EGFR = Epidermal Growth Factor Receptor, HER2 = Human Epidermal Growth Factor Receptor 2, MAPK = Mitogen-activated Protein Kinase, mTOR = Mechanistic Target of Rapamycin, PI3K = Phosphoinositide 3-kinase, SERDs = Selective Estrogen Receptor Down Regulators, TKIs = Tyrosine Kinase Inhibitors, SERMs = Selective Estrogen Receptor Modulators, RAS = Rat Sarcoma Viral Proto-Oncogene, Akt = Serine-threonine Protein Kinase.

effects. For instance, tamoxifen acts as a partial nonsteroidal agonist in some tissues such as the liver, uterus, and bone. However, tamoxifen is a competitive ER inhibitor in breast and brain. Hence, the SERMs are categorized as being "modulators" rather than "down-regulators" of ER or estrogen signaling (Powles, Ashley, Tidy, Smith, & Dowsett, 2007).

Tamoxifen is metabolized by cytochrome P450 (CYP450) enzymes into active metabolites endoxifen and 4-hydroxytamoxifen (4-HT), which then bind to ER and suppress the proliferation of ER-positive breast cancer cells (Lumachi, Brunello, Maruzzo, Basso, & Basso, 2013; Wu, X. et al., 2009). The SERMs, such as tamoxifen, are extensively used to treat ER expressing breast cancer in both pre- and post-menopausal women (Lumachi, et al., 2013). The selective estrogen receptor down-regulators (SERDs) are different from SERMs, because they function as ER antagonists in all tissues (Yeh, et al., 2013; Lumachi, et al., 2011). Fulvestrant is the only Food and Drug Administration (FDA) approved SERD to treat breast cancer and competes with estrogen for the ligand-binding pocket in the ER, which induces proteolytic downregulation of the ER in breast cancer cells. The affinity of fulvestrant for the ER is 100 times stronger than the affinity of tamoxifen (Wardell, Marks, & McDonnell, 2011). Fulvestrant is efficacious in treating tamoxifen resistant breast cancer, and it is combined with an AI when treating metastatic ER-positive breast cancer (Ikeda, et al., 2011; Jiang, et al., 2014).

1.2.2. HER2-enriched Subtype

The breast cancer subtype with HER2 gene amplification is termed HER2-enriched breast cancer (Prat, et al., 2015). Because HER2-enriched breast cancer has aggressive biological behavior and a poor clinical outcome, its overall survival rate is worse than Luminal A and Luminal B breast cancer subtypes (Burstein, 2005). The HER2 gene encodes a transmembrane protein that plays a vital role in the regulation of cell growth, survival, and differentiation

(Wahler & Suh, 2015; Ménard, Pupa, Campiglio, & Tagliabue, 2003). HER2-enriched tumors have a higher frequency of mutations across the genome compared with Luminal A and Luminal B breast cancer (Neve, Lane, & Hynes, 2001); 72% and 39% of HER2 breast cancers exhibit mutations in TP53 and PIK3CA, respectively (Prat, et al., 2015). The overexpression of HER2 in breast cancer promotes metastatic properties of tumor cells and increases the chances for drug resistance in response to chemotherapy and endocrine therapy (Prat & Baselga, 2008). There are four anti-HER2 drug categories that are used to treat HER2 positive breast cancer; they include 1) monoclonal antibodies (mAbs), 2) small molecule tyrosine kinase inhibitors (TKIs), 3) antibody-drug conjugates (ADCs), and 4) new emerging therapies such as heat shock protein 90 (HSP90) inhibitors.

The epidermal growth factor receptor (EGFR) family of proteins is a group of transmembrane receptor tyrosine kinases that includes HER2, human epidermal growth factor receptor 3 (HER3), and human epidermal growth factor receptor 4 (HER4) (Lv, et al., 2016). The ligands that bind these receptors include epidermal growth factor (EGF), heregulin, and tumor growth factor α (TGFα). Upon activation, HER2 will form a heterodimer with another member of the EGFR family of receptors and induce phosphorylation-mediated signaling pathways that promote breast cancer progression (Tzahar, et al., 1996) (Figure 2). The HER2 monoclonal antibodies trastuzumab and pertuzumab inhibit HER2 signaling by preventing the homodimerization or heterodimerization of HER2 receptor complexes, which inhibits HER2-mediated signaling in breast cancer cells (Hudis, 2007). Trastuzumab has also been shown to induce endocytosis of HER2, leading to its degradation and increases in cellular apoptosis (Hudis, 2007). Even though trastuzumab has extended survival in patients with breast tumors that overexpress HER2, resistance occurs through alterations of HER2-regulated downstream

signaling pathways that are induced by other tyrosine kinase receptors and or shedding of HER2 receptors (Wong & Lee, 2012; Rexer & Arteaga, 2012) (Figure 2).

Small molecule TKIs were developed to overcome HER2 resistance (Figure 2). TKIs bind to the intracellular tyrosine kinase domain of HER2 to prevent the phosphorylation of tyrosine kinase, which inhibits the activation of signaling pathways that are downstream of HER2 signaling (Lv, et al., 2016). One example is lapatinib, the second FDA approved anti-HER2 targeting agent. Lapatinib is an orally active, dual small molecular inhibitor of HER2 and human epidermal growth factor receptor 1 (HER1) that induces prolonged inhibition of tyrosine phosphorylation in tumor cells (Johnston & Leary, 2006; Nahta, Yu, Hung, Hortobagyi, & Esteva, 2006). A previous study found that lapatinib had a high efficacy in halting the progression of HER2 expressing breast cancer when used as a first line therapy, where 24% of patients demonstrated an increase in overall response and progression-free survival rate (Gomez, et al., 2008). The mechanism of action for lapatinib has not been determined; however, there are several hypotheses to explain how the drug suppresses HER1 and HER2 (Tai, Mahato, & Cheng, 2010). The first hypothesis is that lapatinib specifically targets the tyrosine kinase domain of HER2 and HER1 to inhibit downstream signaling (Xia, et al., 2002). The second hypothesis proposes that lapatinib perturbs the activity of signaling proteins that sit downstream of HER2 (Xia, et al., 2002). The third hypothesis is that lapatinib induces breast cancer cell apoptosis by inhibiting insulin-like growth factor-1 receptor (IGF-1R). Lapatinib, in combination with HER2 monoclonal antibodies has been shown to induce a synergistic effect in the treatment of HER2-enriched breast cancer (Konecny, et al., 2006; Xia, et al., 2005). The observed synergistic effect of combining lapatinib with a HER2 blocking antibody has been attributed to differences in the mechanism by which lapatinib and HER2 blocking antibodies target HER2 (Xia, et al., 2005).

ADCs are a newer class of HER2-targeting agents, where the monoclonal antibody is conjugated with a cytotoxic agent through a thioester linker. Ado-trastuzumab emtansine (T-DM1) is an ADC that was FDA approved in 2013 (Amiri-Kordestani, et al., 2014). The mechanism of action has been postulated to initially start with trastuzumab binding specifically to the extracellular portion of HER2. Binding between trastuzumab and HER2 is postulated to induce a form of passive endocytosis of HER2-trastuzumab complexes. These HER2-drug complexes enter the cytoplasm, followed by their degradation by the lysosome, which then releases the emtansine (DM1) component from the complex via cleavage of the thioester linker and allows DM1 to exert its cytotoxicity by inhibiting microtubule formation, inducing cell cycle arrest and promoting apoptosis (Martínez, et al., 2016; Lewis Phillips, et al., 2008).

Other emerging HER2 targeting agents include HSP90 inhibitors. The stability and function of several cellular proteins is dependent on the molecular chaperon function of HSP90 (Chen, Singh, & Perdew, 1997). Consequently, HSP90 inhibitors will induce the degradation of various cytosolic transcription factors but is postulated to be very effective at inhibiting breast cancer progression because breast cancer cells exhibit high levels of HSP90 (Solit, et al., 2002; Pick, et al., 2007). HER2 stability is dependent on HSP90. Therefore, the inhibition of HSP90 reduces the stability of HER2, which in turn reduces the survival of HER2 positive breast cancer cells (Huszno & Nowara, 2016; Munagala, Agil, & Gupta, 2011). The first generation HSP90 inhibitor tanespinmycin, has reported anti-tumor activity in HER2-enriched breast cancer and is currently undergoing clinical trials in combination with trastuzumab (Tsang & Finn, 2012).

1.2.3. Triple Negative Subtype

TNBC is an aggressive breast cancer subtype that lacks the expression of the ER, PR, and HER2 receptors. Because there are no specific molecular targets for the treatment of TNBC,

women with this disease have high rates of metastatic breast cancer and breast cancer recurrence (Zhang, Liu, Wang, & Zhang, 2016; Ismail-Khan & Bui, 2010). The only option for women with TNBC is systemic chemotherapy that comes with inherent toxicities (Figure 3). TNBC is correlated with a high risk for relapse and poor overall survival compared to the other breast cancer subtypes. The high rate of TNBC relapse is postulated to be attributed to the lack of drugs that are specific for TNBC (Zhang, Liu, Wang, & Zhang, 2016). Clinical studies, however, have demonstrated that TNBC is initially responsive to chemotherapy. Indeed, a meta-analysis of five adjuvant trials was conducted to compare anthracycline-containing regimens which include: cyclophosphamide, methotrexate, and fluorouracil (CMF). The results of the study showed that anthracycline-containing regimens were superior to CMF for the treatment of TNBC (Di Leo, et al., 2010). Therefore, anthracycline/taxane-based adjuvant regimens are currently the most rational therapy for women with TNBC. Although TNBC is initially responsive to chemotherapy, prolonged therapy with these drugs usually leads to drug-resistance. Despite initial responses to anthracycline/taxane-based regimens, women with TNBC often relapse and tumors return more drug resistant and aggressive. Therefore, the identification of TNBC molecular targets will be important for improving the survival of women with this disease.

To find a solution for TNBC relapse and drug-resistance, Burstein et al. identified 4 stable characterized subgroups of TNBC to help identify any potential molecular targets and novel treatment strategies (Table 1): 1) Luminal/Androgen Receptor (LAR), 2) Mesenchymal (MES), 3) Basal-like Immune Suppressed (BLIS) and 4) Basal-like Immune Activated (BLIA) (Burstein, et al., 2015). The identification of novel molecular targets is critical for improving patient survival with TNBC and must be fundamentally different than what is currently being used (Zhang, Liu, Wang, & Zhang, 2016). Novel agents for TNBC that are currently being

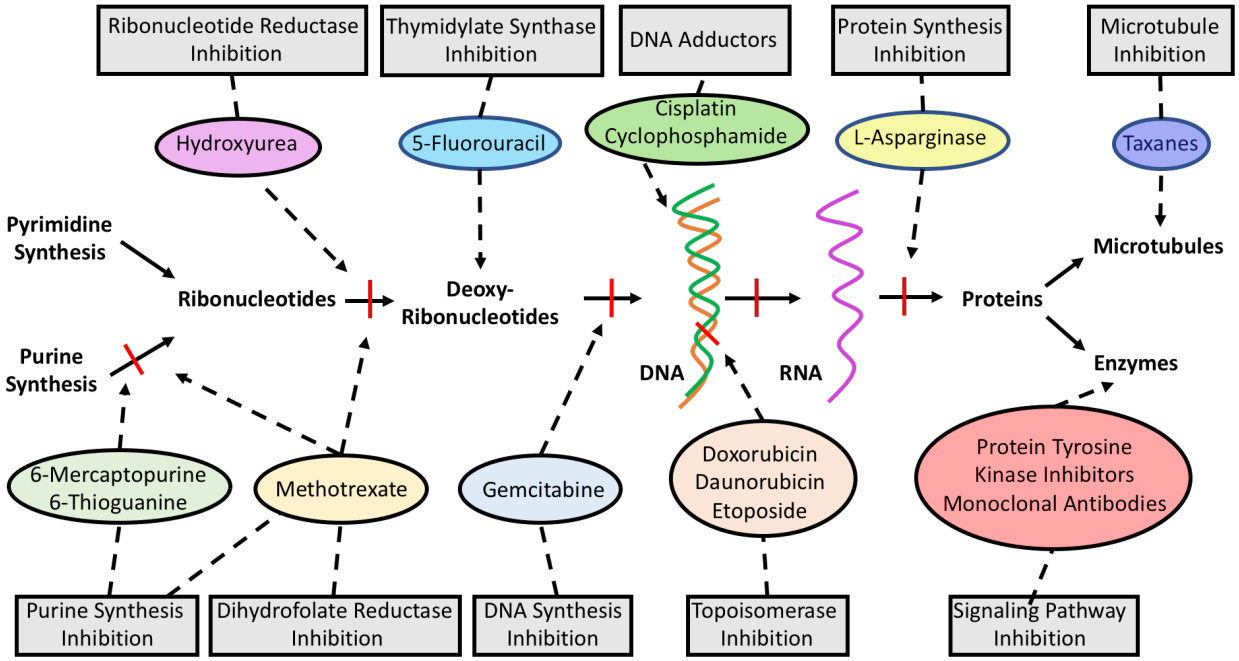


Figure 3. Current Systemic Chemotherapy Agents that are Used in the Treatment of Triple Negative Breast Cancer

TNBC Subtype	Prognostic Status	Gene Expression Unique to TNBC Subtype
Luminal/Androgen Subtype (LAR)	• **Gene expression exhibits ER, AR, and HER4 signaling** • **Demonstrates expression of ER and other ER-regulated genes** • **May respond to anti-estrogen and -androgen therapies in conjunction with chemotherapies**	• **ER: Estrogen Receptor** • **CAXII: Carbonic Anhydrase 12** • **AR: Androgen Receptor** • **RET: Ret Proto-oncogene**
Mesenchymal Subtype (MES)	• **Previously described as "stem-like" or "claudin-low"** • **Expresses genes normally exclusive to osteocytes and adipocytes** • **May respond to PPARγ-inhibitors in conjunction with chemotherapies**	• **ADH1β: Alcohol Dehydrogenase 1β** • **GHR: Growth Hormone Receptor** • **PPARγ: Peroxisome Proliferator-activated Receptor γ** • **IL1R1: Interleukin 1 Receptor, Type I**
Basal-like Immune Suppressed (BLIS)	• **Exhibits downregulation of immune- and cytokine-regulation pathways** • **Exhibits the worst clinical outcome of TNBC subtypes** • **May respond to immune-checkpoint inhibitors in conjunction with chemotherapies**	• **VTCN1: V-set Domain Containing T-cell Activation Inhibitor I** • **TUBB2B: Tubulin β, Class IIB** • **KIT: KIT Proto-oncogene Receptor Tyrosine Kinase** • **FGFR2: Fibroblast Growth Factor Receptor 2**
Basal-like Immune Activated (BLIA)	• **Displays up-regulation of genes controlling immune function** • **Exhibits the best clinical outcome of TNBC subtypes** • **May respond to cytokine- or immune-checkpoint inhibitors in conjunction with chemotherapy**	• **CXCL10: C-X-C Motif Chemokine Ligand 10** • **CTLA4: Cytotoxic T-lymphocyte-associated Protein 4** • **TOP2α: Topoisomerase 2α** • **LCK: Lymphocyte-specific Protein Tyrosine Kinase**

Table 1. The Novel Subtypes of Triple Negative Breast Cancer
The novel subtypes of TNBC along with prognostic factors and some selected genes with significant overexpression unique to each subtype that can serve as potential drug targets**.** 1) LAR, 2) MES, 3) BLIS, and 4) BLIA. Using independent TNBC datasets, Burstein et al. determined that BLIS and BLIA tumors have the worst and best prognoses, respectively (independently of other known prognostic factors), compared to the other subtypes. Collectively, RNA and DNA genomic studies identified stable, reproducible TNBC subtypes characterized by specific RNA and DNA markers, which can help identify potential targets for more effective treatments of TNBC cells. TNBC subtype classification is also based on molecules they do express as opposed to molecules they do not express. All molecules shown in this table have the potential to be targeted for treatment or are already undergoing clinical trials.

studied include poly-(adenosine diphosphate (ADP)-ribose) polymerase (PARP) inhibitors, cytotoxic T-lymphocyte-associated protein 4 (CTLA4) inhibitors and androgen receptor (AR) inhibitors (Burstein, et al., 2015).

PARPs are a large family of enzymes that are important for the repair of single-stranded breaks in deoxyribonucleic acid (DNA) through the base excision repair pathway and were identified to be a potential target for the treatment of the MES subtype in TNBC (Burstein, et al., 2015; Hoeijmakers, 2001). When certain chemotherapies cause DNA damage, PARP repairs the damage and this in turn promotes drug resistance (Figure 4). Because DNA contains various lesions such as single-strand breaks, double-stranded breaks, and homologous recombination, inhibiting PARP leads to the accumulation of double stranded breaks; resulting in genomic instability and eventually cell death (Amé, Spenlehauer, & de Murcia, 2004). Therefore, PARP inhibitors are postulated to increase the sensitivity and responsiveness of TNBC to chemotherapy. Currently, there are several PARP inhibitors in clinical development. The combination treatment of PARP inhibitors with chemotherapy in TNBC has been investigated in phase I-III clinical trials, and this combination showed a favorable clinical response. One example is a trial that showed the combination of carboplatin and PARP inhibitor, veliparib, added to weekly paclitaxel and followed by doxorubicin and cyclophosphamide led to a doubling of the complete response rate in patients with TNBC from 26% to 52% (Rugo, Olopade, DeMichele, & Yau, 2016). However, long-term treatment with PARP inhibitors resulted in drug resistance by increasing expression of P-glycoprotein (P-gp) efflux pumps (Rottenberg, et al., 2008).

Prior reports have established that breast cancer cells are able to initiate an immune response that increases the population of tumor infiltrating lymphocytes (TILs) into breast

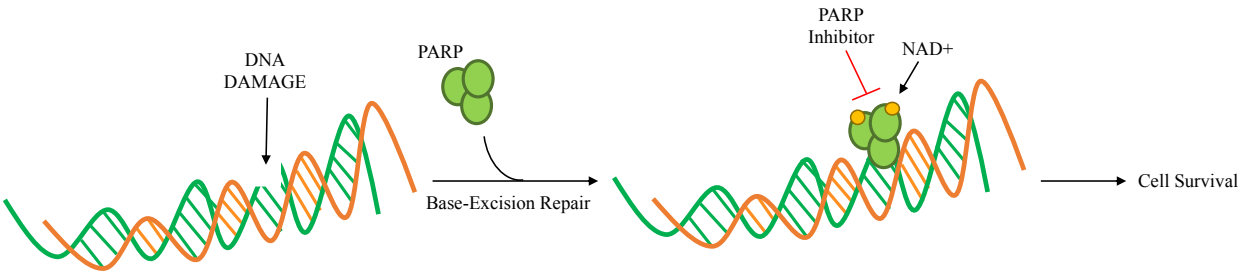


Figure 4. PARP Mechanism and Inhibition
The PARPs have a particularly critical role in the base-excision repair pathway, binding to single-strand breaks in DNA, modifying proteins in the vicinity, and ultimately leading to the recruitment of DNA repair proteins to the sites of damage. PARP inhibitors block the activity of the PARP enzymes by mimicking the nicotinamide moiety of NAD+ and binding to the PARP catalytic site, which either directly blocks PARP enzymatic activity or causes PARP to accumulate on DNA and promotes apoptosis. NAD+ = Nicotinamide Adenine Dinucleotide, PARP = Poly-(ADP-ribose) Polymerase.

tumors (Disis & Stanton, 2015). Increasing the number of TILs in breast tumors was correlated with a favorable treatment outcome depending on the intensity of the tumor immune response (Demaria, et al., 2001). CTLA4 is an immune checkpoint ligand found on the surface of T-cells that modulates the generation and maintenance of immune responses (Gross & Jure-Kunkel, 2013). CTLA4 is critical for inhibiting T-cell immune response by regulating cell cycle progression and maintains a favorable tumor microenvironment (Greenwald, et al., 2002). The monoclonal antibody tremelimumab, which inhibits the CTLA4 pathway, was evaluated in hormone-positive breast cancer and has shown to abrogate immunosuppressive activity (Cimino-Mathews, Foote, & Emens, 2015). Targeting CTLA4 effectively promotes T-cell activity, thereby potentiating the antitumor immune response (Chawla, Philips, Alatrash, & Mittendorf, 2014). Therefore, immunomodulatory drugs can lay the foundation for immune-based therapies in TNBC, especially for the BLIA subtype, which was identified to express higher levels of CTLA4 compared to the other TNBC subtypes (Burstein, 2005).

It has also been shown that the LAR subtype of TNBC represented a unique type of breast cancer with adverse clinical outcome. As a result, AR inhibition was regarded as a potential endocrine therapy for patients with this particular subtype of TNBC (Choi, Kang, Lee, & Bae, 2015). The LAR subtype characterized by AR expression was shown to be sensitive to the AR antagonist bicalutamide alone or in combination with PI3K inhibitors (Lehmann, et al., 2011; Burstein, 2005; Crumbaker, Khoja, & Joshua, 2017). These new findings suggest that targeting the AR in TNBC could be beneficial.

1.2.4. Breast Cancer Stem Cells

The discovery of BCSCs came from a flow cytometry experiment that identified a small subpopulation (<1% of the tumor population) of cancer cells which expressed higher cluster of

differentiation 44 (CD44), lower cluster of differentiation 24 (CD24) and increased aldehyde dehydrogenase 1 (ALDH1) phenotype (i.e. $CD44^{+}CD24^{low/-}ALDH1^{+}$) (Pires, Amorim, Souza, Rodrigues, & Mencalha, 2016). This $CD44^{+}CD24^{low/-}ALDH1^{+}$ population of cells was highly tumorigenic, because transplanting a few hundred of them into mice was sufficient to regenerate a breast tumor (Al-Hajj, Wicha, Benito-Hernandez, Morrison, & Clarke, 2003). Extensive evidence shows that BCSCs are associated with tumor initiation, cancer progression, metastasis, and resistance to chemotherapy. Furthermore, they are also associated with tumor relapse and treatment failure (Al-Ejeh, et al., 2011). Therefore, a better understanding of how BCSCs contribute to breast cancer progression may lead to better cancer therapies (Figure 5). Analogous to normal stem cells, BCSCs are reliant on embryonic pathways to maintain their capacity for pluripotency and self-renewal. The pathways that control these stem cell features include the wingless-type MMTV integration site family (Wnt)/β-catenin, Hedgehog (Hh), and Notch (Pires, Amorim, Souza, Rodrigues, & Mencalha, 2016). These signaling pathways not only maintain cancer stem cell features, they also allow tumor cells to thrive in hypoxic environments (Figure 6). The roles of Wnt, Hedgehog and Notch pathways in breast cancer will be reviewed in the following sections.

The Wnt/β-catenin signaling pathway plays a vital role in developmental processes that determine cell patterning and cell fate determination (Howe & Brown, 2004). Wnt-protein ligands are palmitoylated by porcupine, released from cells, and exert their cellular effects by binding to cell surface Frizzled receptors that are complexed with low-density lipoprotein 5 (LRP5) and low-density lipoprotein 6 (LRP6) (Figure 6). The Wnt-Frizzled-LRP5-LRP6 receptor complex attracts axin and dishevelled (DVL) to the cell membrane, which triggers the inhibition of glycogen synthase kinase-3β (GSK-3β). Inhibition of GSK-3β activity in response

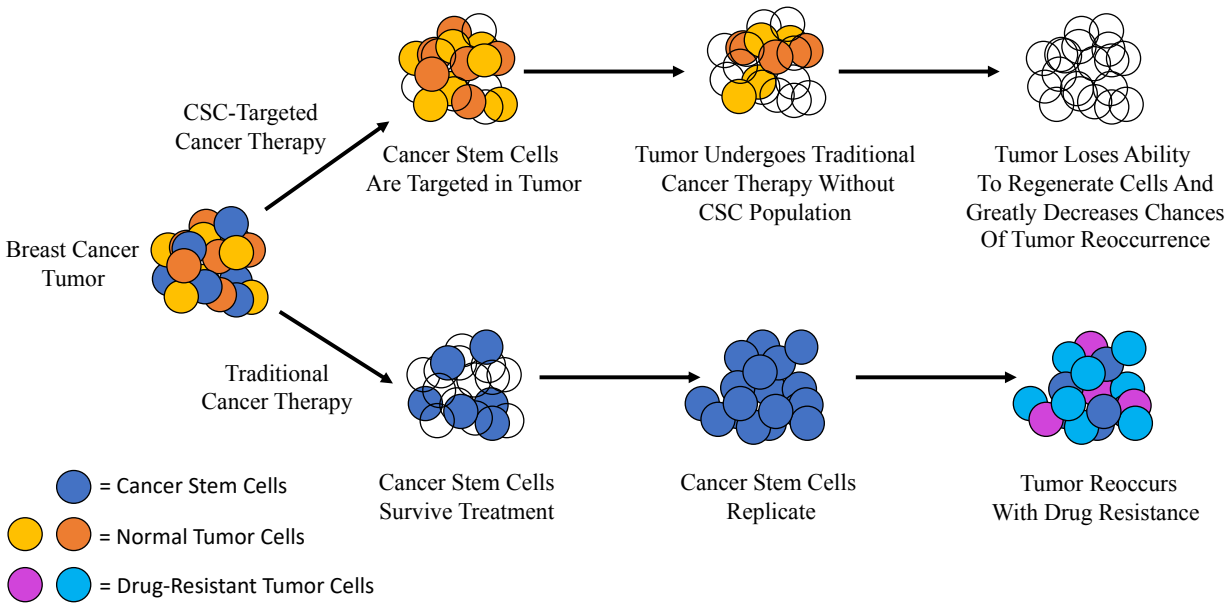


Figure 5. The Strategy Behind Targeting the Breast Cancer Stem Cell Population in Breast Tumors

Targeting the stem cell niche to eradicate BCSCs represents a new area of therapeutic development. Understanding the biology of BCSCs and the mechanisms that support them in breast cancer could help improve tumor treatment and prevent recurrence and metastasis. The theory behind targeting CSCs in breast tumors is to prevent tumor recurrence and drug resistance. BCSCs = Breast Cancer Stem Cells, CSCs = Cancer Stem Cells.

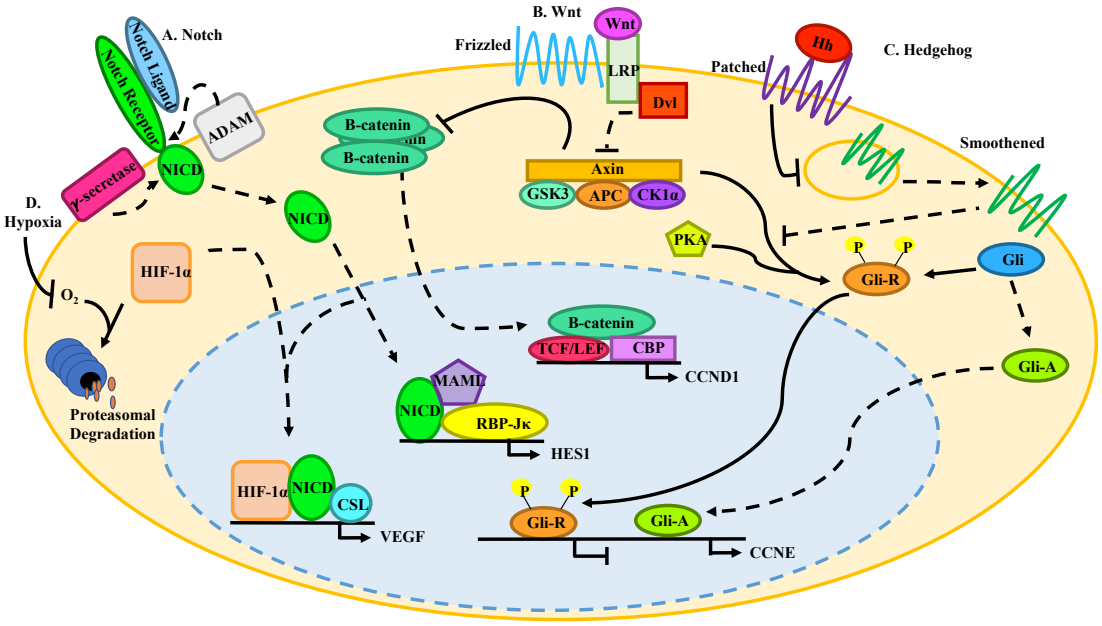


Figure 6. The Activation of Developmental Signaling Pathways in Cancer Stem Cells
Notch, Wnt, and Hedgehog signaling pathways regulate normal and stem cell fate. (A) Notch signaling. Upon binding of Notch ligands to the Notch receptor, the NICD translocates to the nucleus where it binds with the CSL co-activator complex and MAML to induce transcription of its target genes for stem cell dedifferentiation. (B) Wnt signaling. Canonical Wnt signaling occurs through the stabilization and nuclear accumulation of β-catenin. Wnt ligands bind to the Frizzled receptor and the LRP co-receptor, activates DVL and recruits the axin degradation complex to the LRP receptor away from β-catenin, preventing its degradation. β-catenin is then free to translocate to the nucleus and associate with the transcription factors TCF/LEF and CBP to initiate transcription of target genes for mammary gland development and stem cell renewal. (C) Hedgehog signaling. Hh is a secreted ligand that binds to its receptor, Patched. When Patched is activated by Hh binding, inhibition of the SMO receptor is relieved, which allows SMO to localize to the plasma membrane. SMO is then able to inhibit the phosphorylation and cleavage of Gli which prevents the formation of Gli-R and instead promotes the formation of Gli-A, which translocates into the nucleus and initiates transcription of target genes for stem cell regulation. (D) In hypoxia, HIF-1α is not phosphorylated by PHD proteins and is stabilized in the cytoplasm by NICD, where it is then translocated into the nucleus and induces gene expression for maintaining undifferentiated cancer stem cells. JAG1 = Jagged1, DLL1 = Delta-like ligand 1, ADAM = A-disintegrin and metalloproteinase, NICD = Notch Intracellular Domain, HIF-1α = Hypoxia Inducible Factor 1α, RBP-Jκ = Recombination Signal Binding Protein for Immunoglobulin Kappa J Region, CSL = RBP-Jκ/Suppressor of Hairless/ Lag-1, MAML = Mastermind-like, LRP = Low-Density Lipoprotein Receptor, DVL = Dishevelled, GSK-3β = Glycogen Synthase Kinase-3β, APC = Adenomatous polyposis coli, CK1α = Casein Kinase 1α, TCF/LEF = T-cell Factor/Lymphoid Enhancing Factor, CBP = cAMP Binding Protein, PKA = Protein Kinase A, Hh = Hedgehog, SMO = Smoothened, Gli = glioma associated oncogene, Gli-R = Restricted Gli, Gli-A = Activated Gli, PHD = Prolyl Hydroxylase Domain, CCNDI = Cyclin D1, VEGF = Vascular Endothelial Growth Factor, CCNE = Cyclin E, HES1 = Hairy Enhancer of Split 1. Dashed Arrows = Post-activation.

to a Wnt ligand promotes the accumulation of β-catenin. The increase in β-catenin promotes its translocation into the nucleus at which point it functions as a transcriptional activator by binding with cyclic adenosine monophosphate (cAMP) response element binding protein (CREB) (also known as CREB binding protein (CBP)) and T-cell factor/lymphoid enhancing factor (TCF/LEF) transcription factors. The β-catenin-CBP-TCF/LEF complex then increases the transcription of oncogenes such as v-myc avain myelocytomatosis viral oncogene homolog (c-myc), CCND1, and Wnt (Howe & Brown, 2004; Macdonald, Semenov, & He, 2007). The Wnt signaling pathway is constitutively activated in BCSCs by an autocrine mechanism and is associated with the maintenance of stem cell properties (Giles, van Es, & Clevers, 2003; Jang, et al., 2015). The importance of Wnt signaling in CSCs was determined in a study where inhibition of Wnt signaling altered the stem cell phenotype (i.e. $CD44^{+}/CD24^{-}/ALDH1^{-}$), which resulted in the reduction of in vitro and in vivo tumor formation and cell migration (Jang, et al., 2015; Zhao, et al., 2016; Kim do, et al., 2016).

The Hedgehog (Hh) family is comprised of three members: Sonic hedgehog (Shh), Indian hedgehog (Ihh) and Desert hedgehog (Dhh). The Hedgehog signaling pathway controls embryonic development by regulating cell proliferation, cell fate determination and cell patterning (Chen, Wilson, & Chuang, 2007). The Hedgehog signaling pathway has also been identified to play an important role in maintaining the stem cell population in adult animals (Justilien & Fields, 2015). There are three Hh zinc finger protein transcription factors that are sequestered in the inactive state in the cytoplasm with kinesin family member 7 (KIF7) and suppressor of fused (SUFU): glioma associated oncogene-1 (Gli-1), glioma associated oncogene-2 (Gli-2), and glioma associated oncogene-3 (Gli-3). Smoothened (SMO) is located in the cytosol and is responsible for transduction of Hh signaling inside the cell by regulating the

activation of the Gli transcription factors (Chen, Wilson, & Chuang, 2007). In the inactive Hh pathway, patched1 (PTCH1) blocks the activation and migration of SMO. In cancer cells, Hh is released into the tumor microenvironment and enhances stromal cell activity. Once Hh is secreted, it binds to PTCH1 on adjacent cells and allows for SMO to activate signal transduction at the cell membrane (Figure 6). Activated SMO undergoes phosphorylation by casein kinase 1α (CK1α) and protein kinase A (PKA), then removes the Gli transcription factors away from the KIF7-SUFU repressor complex (Chen, Wilson, & Chuang, 2007) (Figure 6). The Gli transcription factors are released and travel to the nucleus to activate transcription of Hh target genes (Lum & Beachy, 2004). There are several pieces of evidence showing that Hh signaling is important for breast cancer biology. One example being that overexpression of Hh or PTCH1 mutations results in constitutively active SMO, which causes increased Gli activation (Tao, Mao, Zhang, & Li, 2011). Furthermore, these alterations have been demonstrated in the more aggressive breast cancer subtypes, such as TNBC (Tao, Mao, Zhang, & Li, 2011) and is essential for promoting growth and self-renewal, not just in BCSCs but also in stem cells from other tumors (Tanaka, et al., 2009; Liu & Micha, 2010; Wicha, Clarke, & Simeone, 2007; Su, et al., 2012).

The Notch signaling pathway controls self-renewal and asymmetric division of normal stem cells; however, its reactivation in epithelial cells contributes to tumorigenesis in the early stages of cancer (D'Angelo, et al., 2015). Notch signaling is predominately activated via cell-to-cell interactions through the binding of one of the four Notch receptors (Notch1, Notch2, Notch3, and Notch4) expressed on the cell surface with one of five Notch ligands (Jagged1 (JAG1), Jagged2 (JAG2), Delta-like ligand 1(DLL1), Delta-like ligand 3 (DLL3), and Delta-like ligand 4 (DLL4)) that are expressed on the cell surface of the signal receiving cell (Bray, 2006)

(Figure 7). The Notch ligand and Notch receptor interaction complex promotes the release of the Notch intracellular domain (NICD) via cleavage by A-disintegrin and metalloproteinase (ADAM) proteases and by the enzymatic complex γ-secretase (Figure 7). Once cleaved, NICD is released into the cytoplasm and translocates into the nucleus where it binds with co-activators Mastermind-like (MAML) and p300 to induce transcription of Notch target genes (Bray, 2006; Ilagan & Kopan, 2007). It was reported that Notch1 expression in breast epithelial cells is enriched in BCSCs that express the $CD44^{+}CD24^{low/-}ALDH1^{+}$ phenotype (Clarke, Anderson, Howell, & Potten, 2003) and was confirmed by D'Angelo et al. The work by D'Angelo et al. demonstrated that breast cancer cells that exhibit high levels of Notch signaling are capable of initiating the formation of mammospheres in cell culture, which is a hallmark feature of BCSCs (Sansone, et al., 2007a; D'Angelo, et al., 2015). Collectively, these results indicate that BCSCs exhibit high levels of Notch signaling that promote their stem cell like activity.

Hypoxia is a hallmark of solid tumors, where the blood supply is not sufficient to meet the needs of the growing tumor (Déry, Michaud, & Richard, 2005). It is known that hypoxic tumors are indictors of poor prognosis and promotes treatment resistance to both radiation and chemotherapy (Qiang, et al., 2012). Hypoxia Inducible Factor 1α (HIF-1α) is translocated to the nucleus where it dimerizes with the aryl hydrocarbon receptor nuclear translocator (ARNT) and forms an active heterodimeric complex that regulates the expression of several genes responsible for promoting cell survival in a hostile environment, such as low oxygen levels, high acidity, and scarce nutrition (Hu, et al., 2014) (Figure 6). Interestingly, hypoxic conditions have been postulated to drive the formation of BCSCs (Helczynska, et al., 2003). The phenomenon occurs through a HIF-1α-NICD interaction, which is promoted by NICD stabilizing HIF-1α when in the absence of ARNT, resulting in the induction of tumor dedifferentiation to breast cancer with

stem cell characteristics (Cejudo-Martin & Johnson, 2005) (Figure 6). This same interaction occurs in embryonic stem cells, which is necessary for the stem cell to maintain an undifferentiated state. Schwab et al. showed that HIF-1α promotes BCSC viability by supporting the formation of mammospheres and maintaining their $CD44^{+}CD24^{low/-}ALDH1^{+}$ phenotype in vivo through the increased expression of cluster of differentiation 133 (CD133), a direct target gene of the Notch pathway (Schwab, et al., 2012). Moreover, it has been shown in vitro that there is a high CD44 expression level in TNBC, which has been shown to be mediated by HIF-1α (Krishnamachary, et al., 2012). The interaction between HIF-1α and the Notch pathway was confirmed by Qiang et al. in glioma stem cells (GSCs) by demonstrating that HIF-1α induced activation of the Notch pathway by binding and stabilizing NICD, driving the maintenance of GSCs (Qiang, et al., 2012). Collectively, the findings of these papers indicate that crosstalk between the Notch signaling pathway and HIF-1α is important for promoting stem cell activity in CSCs under hypoxic conditions.

Inhibitors have been developed that target the embryonic pathways Wnt, Hh, and Notch, based on the rationale that these pathways are not only important for embryogenesis but also important for cancer progression (Figure 7). Regarding the Wnt pathway, LGK974 is a porcupine inhibitor and blocks Wnt palmitoylation and PRI-724 is a CBP/β-catenin antagonist; both have shown promising results and are currently undergoing phase I trials for breast cancer (Liu, et al., 2012; Lenz & Kahn, 2014). The inhibition of Hh signaling has also shown promising results in targeting BCSCs. Salinomycin, a carboxylic polyether ionophore, was shown to be selectively cytotoxic to BCSCs because it reduced Hh signaling by inhibiting the expression of the components necessary for the Hh pathway such as PTCH1, SMO, Gli-1, and Gli-2 (Fu, et al., 2016). This finding has prompted the development of additional Hh inhibitors such as LDE225, a

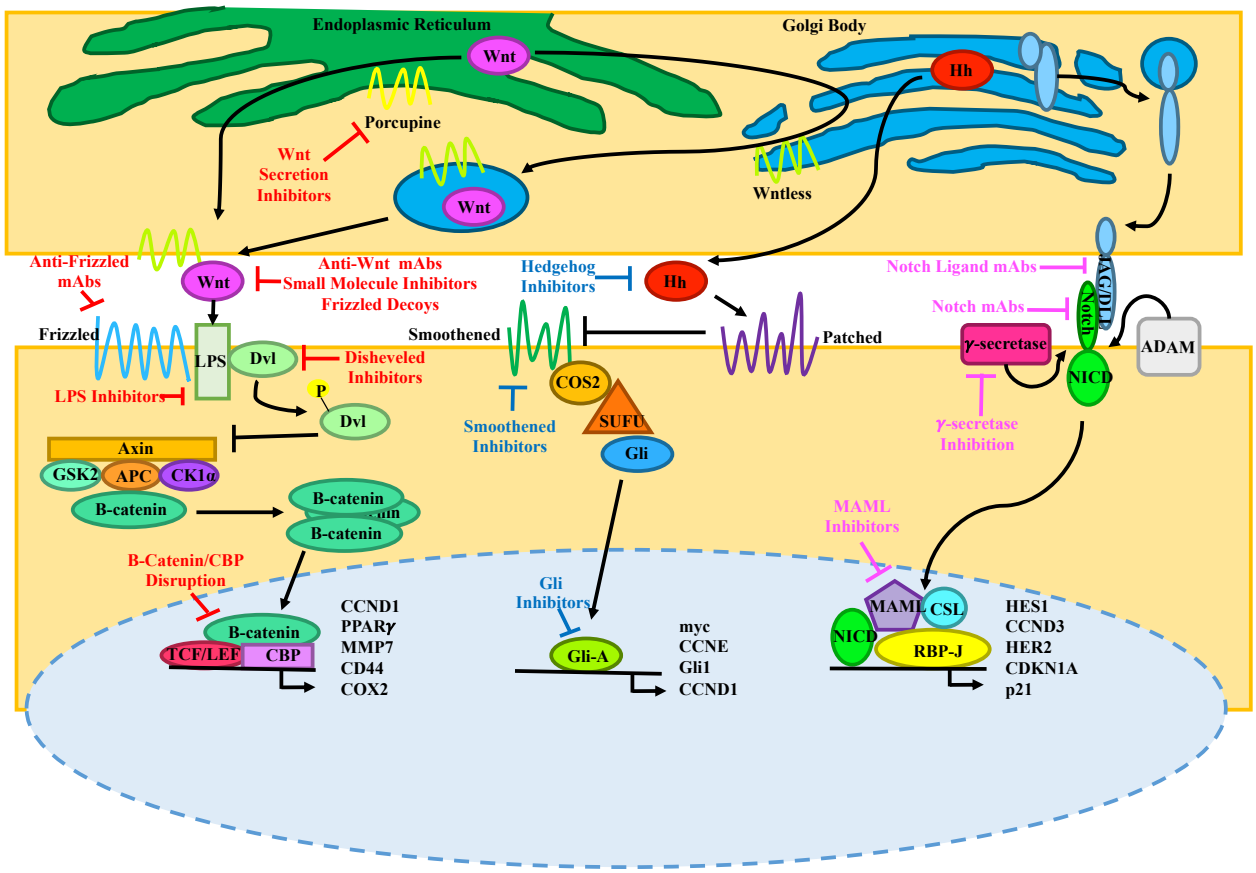


Figure 7. Current Therapeutic Strategies for Targeting Wnt, Hedgehog and Notch Signaling in Breast Cancer Stem Cells

Hedgehog, Notch, and Wnt/β-catenin pathways can be antagonized by molecular targeted agents including ligand-binding molecules, receptor antagonists, ligand receptor antagonists or agents inhibiting intracellular effectors. Red font indicates therapy strategies that target the Wnt signaling pathway, blue font indicates therapy strategies that target the Hedgehog signaling pathway, while purple font indicates strategies for targeting the Notch signaling pathway. Wnt = Wingless-type MMTV Integration Site Family, DVL = Dishevelled, LPS = Lipopolysaccharide, GSK2 = Glycogen Synthase Kinase 2, APC = Adenomatous Polyposis Coli, CK1α = Casein Kinase 1α, CBP = CREB Binding Protein, TCF/LEF = T-cell Factor/Lymphoid Enhancing Factor, COX2 = Cyclooxygenase 2, CD44 = Cluster of Differentiation 44, MMP7 = Matrix Metallopeptidase 7, PPARγ = Peroxisome Proliferator-activated Receptor γ, Hh = Hedgehog, COS2 = Kinesin Motor Protein Costal-2, SUFU = Suppressor of Fused, Gli = Glioma Associated Oncogene, Gli-A = Activated Gli, Gli-R = Restricted Gli, Gli1 = Glioma Associated Oncogene 1, ADAM = A-Disintegrin and Metalloproteinase, NICD = Notch Intracellular Domain, MAML = Mastermind-like, RBP-Jκ = Recombination Signal Binding Protein for Immunoglobulin kJ Region, CSL = RBP-Jκ/Suppressor of Hairless/Lag1, myc = MYC Proto-oncogene, HES1 = Hairy Enhancer of Split 1, HER2 = Human Epidermal Growth Factor Receptor 2, CDKN1A = Cyclin-dependent Kinase Inhibitor 1A, CCND1 = Cyclin D1, CCND3 = Cyclin D3, CCNE = Cyclin E.

SMO inhibitor, which is currently undergoing phase II trials for breast cancer (Irvine, et al., 2016).

More work has focused on the development of Notch inhibitors compared with the development of Wnt and Hh inhibitors. The γ-secretase inhibitors (GSIs) are the most widely recognized Notch inhibitors that are effective against cancer that is resistant to endocrine therapy (Olsauskas-Kuprys, Zlobin, & Osipo, 2013). Anti-estrogen therapy with tamoxifen and fulvestrant increased the activity of BCSCs through activation of the JAG1-Notch4 signaling axis, but when combined with GSI RO4929097 the activity of BCSCs was suppressed (Simões, et al., 2015). The major side-effect to GSIs is gastrointestinal (GI) toxicity due to Notch signaling being vital to maintain the gut epithelium (Wong, et al., 2004). To avoid GI toxicity, new specific antibodies against the Notch receptors and Notch ligands are being developed for better selectivity and thus less toxicity. One example of a Notch receptor antibody targeted the Notch2 and Notch3 receptors (OMP-59R5, tarextumab), which was shown to reduce the breast cancer stem cell population in vivo. Therefore, targeting the stem cell niche to eradicate BCSCs represents a new area of therapeutic development to prevent tumor recurrence and metastasis (Yen, et al., 2015).

1.3. Drug Resistance in Breast Cancer

Despite the complex biological nature of cancer, there have been many recent successes in the treatment of various cancers due to the increased understanding of the diverse molecular mechanisms that modulate tumor development. However, the primary limitation of many cancer drugs is the development of tumors that are drug resistant (Zahreddine & Borden, 2013). Based on the tumor response to the initial therapy, drug resistance in cancer is categorized as either primary or acquired (Meads, Gatenby, & Dalton, 2009; Lippert, Ruoff, & Volm, 2011). While

primary drug resistance exists prior to treatment, acquired resistance occurs after the initial therapy, and unfortunately many patients will at some point develop resistance to treatment (Castells, Thibault, Delord, & Couderc, 2012). Therefore, it is vital to understand the various mechanisms that contribute to drug resistance in breast cancer.

Both primary and acquired resistance can be caused by alterations in drug metabolism, uptake, efflux, and detoxification (Gottesman, 2002). The method of entry into cells depends on the chemical nature of the drug. Some drugs that do not enter the cell use receptors to bind to and activate cell signaling pathways. Other drugs utilize transporters, which allow them to enter the cell and induce cytotoxicity. From this perspective, resistance can result from mutations that alter the activity or reduce the expression of cell surface receptors (Atwood, Chang, & Oro, 2012; Kasper & Toftgård, 2013) or transporters (Galmarini, Mackey, & Dumontet, 2001; Damaraju, et al., 2003) and has been observed with the Hh signaling component SMO, where mutations caused a defective uptake in cyclopamine, a common chemotherapy agent (Yauch, et al., 2009).

Increased drug efflux is promoted by the increased expression of adenosine triphosphate (ATP) binding cassette (ABC) membrane transporters (Gottesman, Fojo, & Bates, 2002). There are three members of the ABC transporter family that have their expression levels correlated with cancer chemo-resistance to various drugs: P-glycoprotein (P-gp), multidrug resistance-associated protein 1 (MRP1), and breast cancer resistance protein (BCRP). P-gp has been identified to transport a wide variety of hydrophobic anti-cancer agents such as vinblastine, doxorubicin, vincristine, and taxol (Gottesman, 2002). MRP1 has been shown to transport natural-products and drugs with a negative charge or that have been modified by glutathione (GSH), glucuronic acid or sulfate (Hipfner, Deeley, & Cole, 1999; Borst, Evers, Kool, &

Wijnholds, 2000), while BCRP has been shown to promote resistance to topoisomerase I inhibitors, anthracyclines, and mitoxantrone (Gottesman, 2002).

To exert cytotoxic effects, many anti-cancer drugs need to undergo metabolic activation (Sampath, et al., 2006). Therefore, drug inactivation also plays a major role in the development of drug resistance. One mechanism involves the conjugation of the drug to reduced GSH, a powerful anti-oxidant that protects the cells against the damaging effects of reactive oxygen species (ROS) (Wilson, Johnston, & Longley, 2009). GSH has been seen to conjugate with platinum-based drugs, which makes them susceptible substrates for ABC transporters and enhances their efflux (Meijer, et al., 1992). Other anti-cancer agents undergo inactivation via phase I drug metabolism, such as the topoisomerase I inhibitor irinotecan, which becomes inactivated by CYP450s. Metallothionein (MT) is a cysteine rich protein that acts like a chelator for various metals and has also been identified to bind platinum-based drugs as another means of drug inactivation (Kasahara, et al., 1991).

Many cancers develop dependency on a particular oncogene, providing a basis for the development of targeted therapies**.** A primary example of this is endocrine therapy, which utilizes SERMs like tamoxifen, SERDs like fulvestrant, and AIs. Resistance to endocrine therapies are seen in 50%-60% of early breast cancer cases and eventually develops in almost all patients with advanced disease (Davies, et al., 2011). There are several mechanisms of resistance to endocrine therapy including loss or modification of ER expression, epigenetic regulation of ER expression and crosstalk between ER and different signaling pathways (Ali & Coombes, 2002; Massarweh & Schiff, 2007). Other causes of drug resistance are due to hypoxia, increased EGFR (Schlessinger, 2004) and HER2 expression (Pietras, et al., 1995), as well as mitogen-activated protein kinase (MAPK) hyperactivation (Ali, Metzger, & Chambon, 1993). However,

crosstalk between ER and different signaling pathways is the main mechanism of endocrine resistance, as targeting one protein alone can become ineffective as other parallel pathways can take over tumor survival. This endocrine crosstalk mechanism was demonstrated in breast cancer cells by Simões et al. who showed that short-term treatment with tamoxifen or fulvestrant decreased breast cancer cell proliferation, but increased BCSC activity through activation of Notch signaling and resulted in tumor recurrence and drug resistance (Simões, et al., 2015). By targeting the Notch pathway via GSIs, Notch signaling was reversed and suppressed BCSC activity that was originally induced by tamoxifen alone (Simões, et al., 2015). Current research shows that inhibiting the Notch signaling pathway via GSIs can decrease drug resistance (Nefedova, Cheng, Alsina, Dalton, & Gabrilovich, 2004) and promote apoptosis (Séveno, et al., 2012) by restoring the effects of tamoxifen and other endocrine targeting agents.

Drug resistance could also result by avoiding apoptotic pathways. Apoptotic resistance can be triggered by inactivating mutations in genes coding for apoptotic proteins (i.e. p53) or by activating mutations in genes coding for anti-apoptotic proteins, such as B-cell lymphoma-2 (Bcl-2) (Evan & Vousden, 2001). Mutations in p53 have been correlated with resistance to doxorubicin treatment in patients with advanced breast cancer (Geisler, et al., 2001). In the case for endocrine therapy, the treatment fails if the balance between proliferation and apoptosis cannot be maintained because the pro-apoptotic genes are increased, and the anti-apoptotic genes are decreased (Ali, et al., 2016). Tamoxifen can act as an ER antagonist in breast cancer cells but in other cell types acts as an ER agonist. As a result, the anti-apoptotic proteins are promoted by estrogen to protect normal cells from cell death, but in the presence of tamoxifen, the pro-apoptotic genes are decreased, and anti-apoptotic proteins are increased (Lewis-Wambi & Jordan, 2009). The tamoxifen resistance is promoted by ER-positive breast cancer via expression

of Bcl-2 (Kumar, Mandal, Lipton, & Harvey, 1996) and tumor growth factor β (TGFβ) (Osborne & Fuqua, 1994). The HER2-enriched breast cancers already have high levels of Bcl-2 which leads to reduced tamoxifen-induced apoptosis. Current research shows that inhibiting the Notch signaling pathway via GSIs can decrease drug resistance and promote apoptosis by restoring the effects of tamoxifen and other endocrine targeting agents (Wang, et al., 2012). Given the importance of Notch signaling in cancer progression, the following chapter will cover the Notch pathway in both cancer and normal physiological functions.

CHAPTER 2

THE NOTCH SIGNALING PATHWAY

The Notch signaling pathway is a highly conserved evolutionary pathway that plays vital roles that have been established as fundamental in both multi-cellular and developmental processes (Borggrefe & Liefke, 2012). The Notch receptor was originally discovered in Drosophila as a neurogenic gene, and its signaling pathways were revealed to be pleiotropic, as it affected many other tissues (Artavanis-Tsakonas, Muskavitch, & Yedvobnick, 1983). These pleiotropic effects and physiological functionalities were identified to hold true in mammalian species, where the Notch signaling pathway was shown to play a pivotal role in embryonic and post-natal development (Andersson, Sandberg, & Lendahl, 2011; Ables, Breunig, Eisch, & Rakic, 2011). More specifically, Notch has been identified to regulate a variety of functional effects, ranging from cell differentiation, cell proliferation, cell survival and apoptosis. It is therefore not surprising that the Notch pathway and its functional effects are exploited in cancer progression (Koch & Radtke, 2010; Ranganathan, Weaver, & Capobianco, 2011).

2.1. Canonical Notch Signaling

When first studied, the Notch signaling pathway appeared to be remarkably simple, as it was activated through direct interactions between the Notch receptors and its ligands on neighboring cells (Figure 8). The mammalian Notch receptor family consists of four Notch receptors (Notch1, Notch2, Notch3, Notch4) and five Notch ligand receptors (JAG1, JAG2, DLL1, DLL3, and DLL4) (D'Souza, Miyamoto, & Weinmaster, 2008). Notch ligands are Delta-Serrate-Lag1 (DSL)-containing type I transmembrane proteins that bind to the Notch receptor and induce a conformational change, exposing the cleavage site on the extracellular domain (ECD) to the ADAM/tumor necrosis factor α (TNFα) converting enzyme (TACE) complex

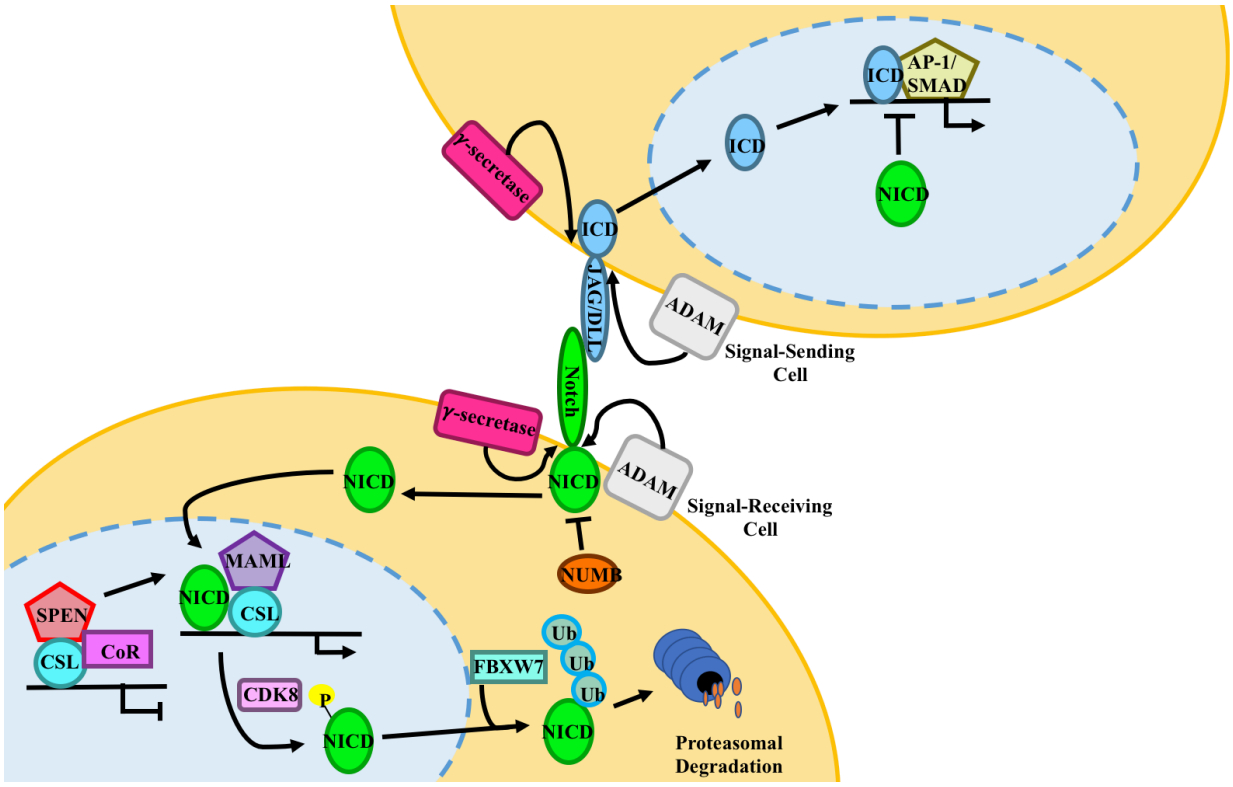


Figure 8. Canonical Notch Signaling
Notch signaling is activated by interaction between the ligand-expressing cell and the Notch-expressing cell, followed by proteolytic cleavage that releases the NICD. Before activation of Notch signaling, CSL is bound to DNA along with CoRs such as SPEN. Upon activation of Notch, the NICD recruits the co-activator MAML and others, and thus converts the CSL-repressor complex into a transcriptional activator complex and drives the transcription of target genes. The signal is terminated by phosphorylation of the NICD, followed by ubiquitylation by FBXW7 and proteasomal degradation. Numb promotes ubiquitylation of the membrane-bound Notch1 and targets the NICD for proteasomal degradation. Signal transduction from Notch ligands also occurs. Proteolytic cleavage releases the ICD of the Notch ligands. The PDZ domain interacts with PDZ-containing proteins, resulting in a signaling cascade. The ICDs of Notch ligands JAG and DLL can also enter the nucleus and regulate transcription, possibly through interactions with AP-1 or the Smad proteins. This transcriptional regulation may be antagonized by the NICD. CoRs = Co-repressors, ICD = Intracellular Domain, PDZ = PSD-95/Dlg/ZO-1, CDK8 = Cyclin-dependent Kinase 8, ADAM = A-disintegrin and Metalloproteinase, FBXW7 = F-Box and WD Repeat Domain Containing 7, NICD = Notch Intracellular Domain, CSL = RBP-Jκ/Su(H)/LAG1, SPEN = MINT and SHARP, MAML = Mastermind-like, Ub = Ubiquitin, AP-1 = Activator Protein-1, p = Phosphate Group.

(Fleming, 1998). The Notch receptor then undergoes another cleavage in the intercellular domain by the presenilin-γ-secretase complex (Jorissen & De Strooper, 2010). This cleavage cascade results in the release of the NICD from the plasma membrane and its translocation to the nucleus (Bray, 2006). Once in the nucleus, the NICD then mediates the conversion of the RBP-Jκ/Suppressor of Hairless (Su(H))/Lag-1 (CSL) repressor complex into a transcriptional activation complex (Wilson & Kovall, 2006), leading to the recruitment of the co-activator mastermind-like (MAML) and the induction of gene expression (Nam, Sliz, Song, Aster, & Blacklow, 2006; Vasquez-Del Carpio, et al., 2011) (Figure 8). Several well-characterized target genes of the Notch pathway have been identified, such as the transcriptional repressors hairy enhancer of split (HES) family and the HES related family basic helix-loop-helix (bHLH) transcription factor with YRPW motif (HEY) family (Borggrefe & Oswald, 2009). Notch signaling is terminated by phosphorylating the NICD, which is targeted for ubiquinylation by F-box and WD repeat domain containing 7 (FBXW7) E3 ligase and degraded via the proteasome (Le Bras, Loyer, & Le Borgne, 2011; Gupta-Rossi, et al., 2001; Le Friec, et al., 2012).

Although the primary role of Notch ligands is to activate Notch signaling, Notch ligands can also have unique Notch-independent functions (Pintar, De Biasio, Popovic, Vanova, & Pongor, 2007). Recent evidence has shown that after binding to the Notch receptor, the DSL-containing ligands also undergo proteolytic cleavage and initiate signaling events that are independent of the NICD (Ikeuchi & Sisodia, 2003). Signaling induction from DSL-containing ligands was determined when ectopic expression of JAG1 transformed rat kidney epithelial cells independently of Notch signaling and required an intact and functional postsynaptic density protein-95 (PSD-95)/discus large homolog (Dlg)/zona occludens 1 (ZO-1) (PDZ)-ligand motif in JAG1; this prompted the hypothesis that the Notch-DSL pathway is bidirectional (Ascano,

Beverly, & Capobianco, 2003). Bidirectional signaling in this case indicates that even though activation of the Notch pathway is receptor-ligand dependent, the intracellular domains (ICDs) of each receptor and ligand regulate transcription independently. JAG1 and other Notch ligands undergo similar processing and proteolytic cleavage to release their respective ICDs. The JAG1 intracellular domain (JICD) has been identified to activate activator protein-1 (AP-1)-mediated transcription (Duryagina, et al., 2013), and inhibit NICD mediated transcription (Metrich, et al., 2015). The delta-like ligand (DLL) intracellular domain (DICD) was also able to mediate TGFβ-Activin signaling through binding to Smads (Hiratochi, et al., 2007), and was also inhibited by the NICD (Jung, et al., 2011). However, even though Notch-independent DSL signaling has been reported and established, its physiological relevance in tumorigenesis has yet to be identified.

The Notch pathway does not act independently. Rather, it is part of a complex network of many signaling pathways that are interconnected (Borggrefe & Liefke, 2012). As an example, the Notch pathway has been identified to be modulated by Wnt signaling (Jin, et al., 2009) and HIF-1α (Gustafsson, et al., 2005). Table 2 identifies various Notch-interacting partners that either inhibit or activate Notch signaling. Within this intricate network are feedback loops, which are essential for establishing limitations of Notch signaling during development (Kim, et al., 2011; Fischer & Gessler, 2007). Notch signaling regulates itself by activating the transcription of regulatory factors that are direct Notch target genes such as hairy enhancer of split 1 (HES1) (Takebayashi, et al., 1994). These regulatory factors are what establish the positive- and negative-feedback mechanisms in Notch signaling, which can be classified based on the stages they act on within the pathway (Figure 9). The first stage that direct feedback regulators act on is the initiation of the Notch response. The genes that code for the Notch receptors, Notch1 and

Notch-interacting Partners		
Interacting Protein	**Partner**	**Notch Signaling Effect**
β-catenin (Jin, et al., 2009)	NICD	+
HIF-1α (Gustafsson, et al., 2005)	NICD	+
JICD (Kim, et al., 2011)	NICD	-
p300 (Oswald, et al., 2001)	NICD/MAML	+
RUNX2 (Ann, et al., 2011)	NICD	-
RUNX3 (Gao, et al., 2010)	NICD	-
Smad (Takizawa, Ochiai, Nakashima, & Taga, 2003)	NICD	+

Table 2. Notch-interacting Partners
There are many interacting factors that can alter the stability and the composition of the activator complex, which precisely regulates the Notch signaling output. HIF-1α = Hypoxia Inducible Factor 1α, JICD = JAG1 Intracellular Domain, RUNX2 = Runt Related Transcription Factor 2, RUNX3 = Runt Related Transcription Factor 3, NICD = Notch Intracellular Domain, MAML = Mastermind-like, - = Notch Inactivation, + = Notch Activation.

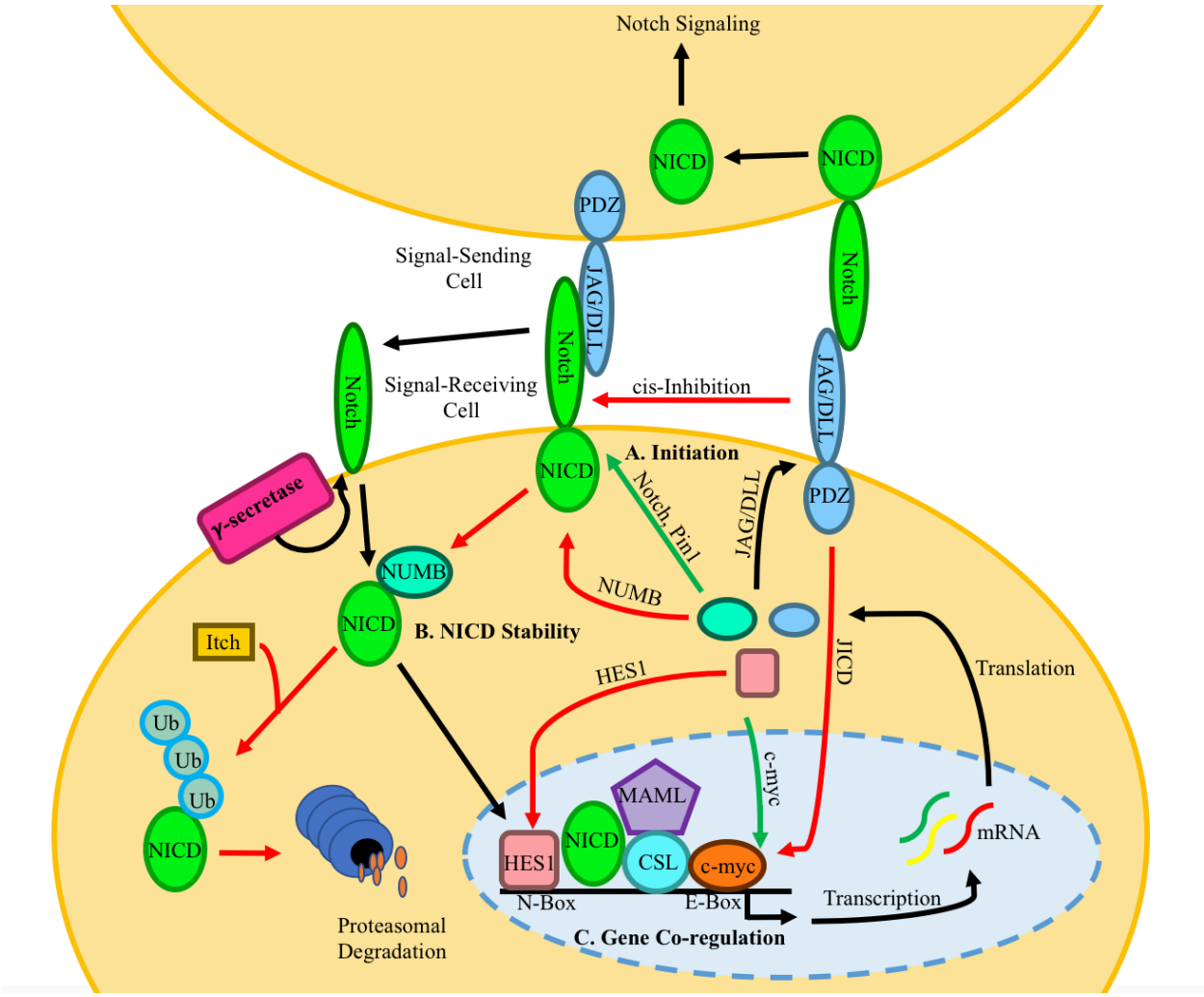


Figure 9. Notch Target Genes that Act as Positive- and Negative-feedback Regulators for the Notch Signaling Pathway

Direct feedback regulators can be classified based on what stages of Notch signaling they can modulate. (A) The initiation phase of Notch signaling (Pin1 and cis-inhibition). (B) NICD and corresponding transcription complex levels and stability (Numb and JICD). (C) Co-regulation gene expression by binding to N-Box (suppressing DNA regions) and E-Box (enhancing DNA regions) sequences of Notch target genes (HES1 and c-myc). NICD = Notch Intracellular Domain, JICD = JAG1 Intracellular Domain, Pin1 = Prolyl-cis/trans Isomerase 1, CSL = RBP-Jκ/Su(H)/Lag-1, MAML = Mastermind-like, HES1 = Hairy Enhancer of Split 1, c-myc = V-myc Avian Myelocytomatosis Viral Oncogene Homolog, PDZ = PSD-95/Dlg/ZO-1, Ub = Ubiquitin. Green Arrows = Positive-feedback Loops, Red Arrows = Negative-feedback Loops.

Notch3, and Notch ligand receptors JAG1 and DLL1 were found to be regulated by the Notch pathway itself (Weng, et al., 2006; Weerkamp, et al., 2006; Chen, et al., 2010; Qian, et al., 2009). The increased expression of these Notch receptors and ligands acts to promote a positive-feedback loop. However, even though increasing expression of these receptors promotes Notch signaling on the adjacent cell, the same receptors act as a negative regulator on the originating cell; a cellular process coined cis-inhibition (del Álamo, Rouault, & Schweisguth, 2011). Nuclear inhibition has been observed through studying the JICD. After the JICD is released from the receptor, it translocates to the nucleus and binds the NICD, where it inhibits Notch signaling by promoting its proteasomal degradation via FBXW7 (Kim, et al., 2011). Therefore, the intracellular functionality of the JICD serves as an additional negative-feedback loop. A form of positive-feedback at the initiation step is increased expression of the Notch target gene prolyl-cis/trans-isomerase 1 (Pin1) (Rustighi, et al., 2009). Once expressed, Pin1 interacts with the Notch1 receptor at the membrane and helps promote cleavage by γ-secretase, which stimulates the release of the NICD. Therefore, increased Pin1 expression results in elevated Notch1 activity and transcriptional output.

Direct feedback regulators also play a role in modulating the amount and stability of NICD. While at the membrane or shortly after release, NICD can interact with Notch target gene product Numb (Figure 9). Numb acts as a negative-feedback regulator by interacting with the NICD and induces ubiquitination via the E3 ligase Itch, which stimulates the ubiquitination and proteasomal degradation of the NICD (Rebeiz, Miller, & Posakony, 2011) (Figure 9). This negative-feedback regulator results in the reduction of the total amount of NICD present in the cell and nucleus, hence reducing its transcriptional output. Runt related transcription factor 2 (RUNX2) also acts as negative regulator by inhibiting Notch1 signaling during osteoblast

differentiation by binding the NICD and inducing its degradation (Ann, et al., 2011). Other Notch target genes are transcription factors that can transmit the Notch signal to further downstream target genes. Some well-known examples of Notch target genes that encode transcription factors include: HES1, hairy enhancer of split 5 (HES5), and HES related family bHLH transcription factor with YRPW motif 1 (HEY1), which are DNA binding transcription factors that act through transcriptional elements to induce or suppress the expression of target genes (Fischer & Gessler, 2007). These enhancer and repressor regions are present in many Notch target genes and there have been several reports showing that Notch target genes can be repressed by HES1, including the HES1 gene itself. This feedback loop was identified as an "incoherent network logic" by Krejcí et al. to signify Notch as a signaling pathway that induces its target genes along with repressors for those target genes, which could serve a purpose to create a temporary window of responsiveness after Notch activation (Krejcí, Bernard, Housden, Collins, & Bray, 2009). Collectively, these positive- and negative-feedback loops play a major role in modulating the Notch pathway and are essential to convert these modulations into versatile biological outcomes.

2.2. Physiological Functions of Notch Signaling: Immunity, Inflammation, Hematopoiesis, and Development

Recent evidence has established that Notch signaling is associated with immunity and inflammation because of its activity in several inflammatory diseases including rheumatoid arthritis (RA) (Ando, et al., 2003), atherosclerosis (Fung, et al., 2007), and primary biliary cirrhosis (Geisler & Strazzabosco, 2015). Expression of Notch receptors and ligands was detected in RA synovial tissues, and excessive activation of Notch1 was observed in synoviocyte cultures from RA patients (Nakazawa, et al., 2001). Therefore, it has been predicted that the

Notch pathway is activated in RA and may modulate the severity of the disease. However, although the association between Notch signaling and inflammation is supported by a copious amount of literature, the mechanisms by which Notch regulates inflammation is poorly understood. Under inflammatory conditions, Notch signaling can be promoted by stimuli from two different sources, exogenous agents such as pathogens and/or endogenous factors such as inflammatory cytokines.

Regarding pathogenic infections, macrophages (Palaga, et al., 2008) and dendritic cells (DCs) (Cheng & Gabrilovich, 2008; Gentle, Rose, Bugeon, & Dallman, 2012; Cheng, Nefedova, Miele, Osborne, & Gabrilovich, 2003) express toll-like receptors (TLRs) that enable them to respond rapidly to coordinate innate and adaptive immune responses. TLRs are a single membrane-spanning, non-catalytic receptor class of proteins that play a key role in the innate immune system and are usually expressed on sentinel cells that recognize structurally conserved molecules, such as macrophages and DCs. Concurrently, macrophages and DCs also constitutively express Notch ligands and receptors on their cellular membrane that respond to Notch signaling (Cheng, Nefedova, Corzo, & Gabrilovich, 2007; Shang, Smith, & Hu, 2016). There is plenty of evidence that TLRs can modulate Notch signaling by inducing expression of Notch receptors as well as Notch ligands JAG1, DLL1, and DLL4 (Fung, et al., 2007; Foldi, et al., 2010; Monsalve, et al., 2009). By enhancing the expression of Notch receptors and ligands, TLR signaling indirectly promotes Notch activation in a manner that is predicted to be dependent on de novo protein synthesis (Palaga, et al., 2008; Hu, et al., 2008). These observations support a binary model where signal 1 represents tonic signaling and signal 2 represents acute TLR signaling (Shang, Smith, & Hu, 2016). This binary model allows us to understand that: 1) resting macrophages show basal levels of the NICD because of constitutive expression of Notch

receptors and ligands, 2) activation of Notch target gene expression occurs rapidly once triggered by TLR stimulation, which circumvents the requirement for activation via receptor-ligand induction, and 3) signal 1 or signal 2 alone is necessary but not sufficient for robust Notch target gene expression in macrophages. Therefore, the cooperation of both signaling pathways is required for optimal activation. However, it is important to note that the mechanism that couples acute TLR signaling to Notch pathway activation is currently unknown.

Inflammatory cytokines such as TNFα and interleukin-1β (IL-1β) are essential for host defense against a plethora of pathogens and are present during innate and immune responses. However, these cytokines can become detrimental and pathogenic under conditions with uncontrolled inflammation and in autoimmune diseases when the expression of these cytokines becomes dysregulated. In RA, it is well established that TNFα plays a key role in RA pathogenesis and is currently validated as a drug target in the treatment of RA. Coincidently, in RA synovial fibroblasts, TNFα induces transcription of Notch1, Notch4, and JAG2 via activation of the nuclear factor-κB (NF-κB)-inhibitor of NF-κB subunit α (IKKα) signaling pathway, which in turn promotes NICD nuclear translocation (Ando, et al., 2003). Another example of TNFα-induced Notch activation is observed in osteoclast precursors, where Notch signaling is activated by TNFα and as a result inhibits osteoclastogenesis and enhances TNFα-mediated inflammatory bone resorption in a feedback manner (Zhao, Grimes, Li, Hu, & Ivashkiv, 2012). Therefore, TNFα appears to function as a promoter of Notch signaling in various cell types. IL-1β is reported to induce expression of Notch target gene HES1 in chondrocytes via Notch1 activation (Ottaviani, et al., 2010). In addition, TGFβ has also been identified to directly induce HES1 expression in several cell types (Ostroukhova, et al., 2006). While there are multiple cytokines that positively regulate Notch signaling, interferon-γ (IFNγ)

functions as a negative regulator of Notch signaling (Hu, et al., 2008) and was observed in human macrophages, where IFNγ suppressed transcription of Notch target genes by TLR and Notch ligands. However, the precise mechanism of how this occurs has not been identified.

Hematopoiesis is the developmental process where pluripotent hematopoietic stem cells (HSCs) produce progeny that undergo proliferation and differentiation as a response to both cell-bound factors and cytokines in a positive and negative manner, which results in the production of mature blood cells of various lineages. The Notch signaling pathway modulates the interactions between HSCs (which express all four Notch receptors) and bone marrow stromal cells (which express various Notch ligands) in the developing immune system (Bigas, Martin, & Milner, 1998). It is important to understand the role of Notch signaling in myeloid cell development and differentiation, even though there are some discrepancies regarding the mechanism(s) involved. One collection of evidence was able to demonstrate that Notch signaling is vital in the maintenance of progenitor cells and blocking terminal differentiation of myeloid cells (Shang, Smith, & Hu, 2016) and was supported when retroviral transduction of activated Notch1 intracellular domain (NICD1) in 32D myeloid progenitor cells inhibited the differentiation of mature granulocytes in response to granulocyte colony-stimulating factor (G-CSF) but not granulocyte macrophage colony-stimulating factor (GM-CSF), without affecting the proliferation of undifferentiated cells. Interestingly, the Notch2 intracellular domain (NICD2) inhibited differentiation of 32D cells in response to GM-CSF but not G-CSF (Milner, et al., 1996; Bigas, Martin, & Milner, 1998), which suggests that even though both Notch1 and Notch2 inhibited myeloid differentiation, there may be independent functions taking place in HSCs depending on the specific differentiation signal involved. Over-expression of HES1 resulted in a similar phenotype and, in addition, also blocked erythroid differentiation (Lam, Ronchini, Norton,

Capobianco, & Bresnick, 2000; Kumano, et al., 2001). On the other hand, Notch has been shown to be required for differentiation of mature myeloid cells. Schroeder & Just were able to demonstrate that conditional expression of the NICD in 32D cells decreased self-renewal and enhanced granulocyte differentiation through JAG1 activity in a transcriptionally active form of RBP-Jκ (Schroeder & Just, 2000).

It was more recently demonstrated by Klinakis et al. that Notch modulated early HSC differentiation in vivo. In a mouse model, Notch signaling was inhibited by targeting γ-secretase complex member nicastrin, and resulted in excessive accumulation of granulocyte and or monocyte progenitors in the blood, spleen, and liver; which is diagnostic of the induction of chronic myelomonocytic leukemia (CMML)-like disease (Klinakis, et al., 2011). Gene expression analysis was able to reveal that the Notch pathway regulates a myelomonocytic-specific gene signature by suppressing gene expression via HES1. Furthermore, in samples from CMML patients, somatic mutations were identified in multiple components of the Notch pathway such as nicastrin, MAML and Notch2, suggesting a potentially tumor-suppressive role for Notch signaling in addition to its involvement in early HSC differentiation (Shang, Smith, & Hu, 2016).

Notch signaling is remarkably pleiotropic. There is not a cell or tissue that is not affected by cell fate choices regulated by Notch signaling (Hori, Sen, & Artavanis-Tsakonas, 2013). With regard to developmental signaling, the consequences of either activating or inhibiting Notch is strictly context-specific. For example, the same Notch signal in one circumstance can promote proliferation while another can result in apoptosis (Hori, Sen, & Artavanis-Tsakonas, 2013). Therefore, how Notch signaling is incorporated with other signaling pathways in the context of a particular cellular physiology will ultimately dictate how Notch activity affects cell fate. Even

though there are a lot of spatial and temporal complexities intertwined within the dynamics of this pathway, cis-inhibition is the hallmark of Notch-regulated cell fate and lateral specification (Greenwald, 1998). Cis-inhibition describes the mechanism by which cells that are in the process of adopting one particular cell fate influence the fate of the neighboring cell. Due to this pathway being so evolutionarily conserved, most eukaryotic lineages rely on cis-inhibition. One classic example of the developmental action of Notch is the case of neuroblast differentiation in Drosophila embryo. In neuroblast differentiation, a cell that is about to become a neuroblast prevents its adjacent neighbors from adopting the same fate and has two important implications to consider when regarding morphogenesis: 1) Notch signaling helps in the separation of specific lineages from a field of developmentally equivalent cells and 2) is a crucial mechanism that specifies borders between cellular fields (Bray, 2006).

Stem cells exist in most embryonic and adult organisms and are defined as cells having the ability for self-renewal. Stem cells also have the ability to generate all cell types of a given organ. During the developmental process, stem cells give rise to all lineages of tissues, but in adults they are responsible for maintaining tissue homeostasis in damaged tissue (Liu, Sato, Cerletti, & Wagers, 2010). Therefore, the balance between self-renewal and differentiation is under tight regulation to allow for proper development to occur and avoid excessive growth, which could potentially lead to cancer. Conditional gain- and loss-of-function studies demonstrated that Notch regulates quiescence and cell cycle exit in neuronal stem cells. Chapouton et al. showed that high Notch activity maintained neuronal stem cells in a quiescent state but inhibiting Notch signaling resulted in increased neuronal stem cell division followed by differentiation, which in turn depletes the neuronal stem cell population (Chapouton, et al., 2010). Once again, this study suggests that cis-inhibition may act as the mechanism that

generated the results. Similar results were obtained in mice with conditional ablation of RBP-Jκ in the adult brain, where all neuronal stem cells differentiated into transit-amplifying cells and neurons. As a result, transient neurogenesis was increased but later depleted the neuronal stem cell population and neurogenesis was completely lost (Ehm, et al., 2010). These results indicate that the Notch pathway is an absolute necessity for the maintenance of neuronal stem cells and for the proper control of neurogenesis in both embryonic and adult brains.

2.3. JAG1-dependent Notch Signaling in Cancer

The Notch pathway is increasingly attracting attention as a new target for cancer treatments considering that ligand-induced Notch signaling has been shown to promote cancer progression (Li, Masiero, Banham, & Harris, 2014). Even though Notch inhibitors such as GSIs are currently undergoing clinical trials and have been extensively researched, targeting Notch ligands provides a more attractive option due to 1) their more restricted expression, 2) better-defined functions and 3) lower mutation rates in cancer. JAG1 is the most extensively researched Notch ligand, is over expressed in various cancer types, has been linked with poor prognosis, and plays a vital role in several functions of tumor biology.

JAG1 is a member of the DSL family that encodes a Type I transmembrane receptor protein. The extracellular portion of this receptor contains a calcium-binding (C2) domain at the N-terminus that binds to the phospholipid bilayer once undergoing glycosylation to modulate JAG1-dependent Notch activation (Cordle, et al., 2008; Whiteman, et al., 2013) (Figure 10). The DSL binding domain is located below the C2 domain which is responsible for binding Notch receptors; this domain was also recently discovered to bind cluster of differentiation 46 (CD46) receptor to regulate T-cell functionality (Le Friec, et al., 2012) (Figure 10). Following the DSL binding domain are multiple highly conserved EGF repeats (human JAG1 consists of 16 EGF

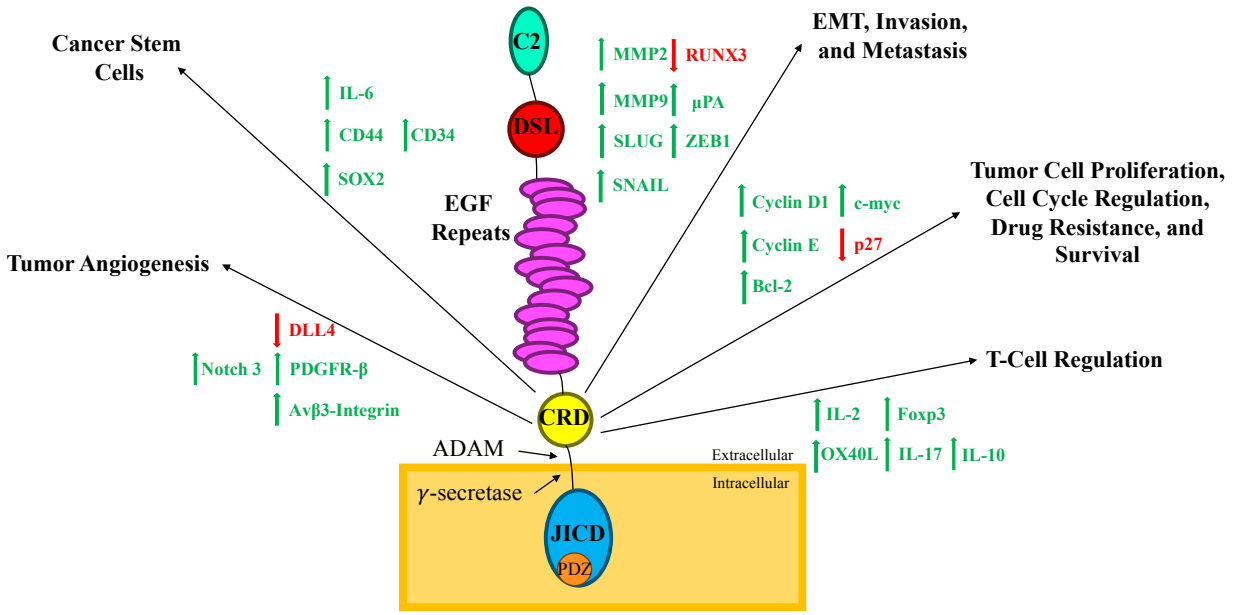


Figure 10. The JAG1 Ligand Receptor and its Roles in Cancer Biology
JAG1 is a type I transmembrane receptor protein. The extracellular portion of the receptor consists of a C2 phospholipid recognition domain, DSL-binding domain, 16 EGF repeats and a CRD. JAG1 can be cleaved by ADAM upon binding to the NOTCH receptor and then undergoes a second cleavage by γ-secretase to release the JICD. The JICD contains a PDZ-ligand binding motif that is responsible for the intrinsic bidirectional signaling induced by JAG1. In cancer and/or in stromal cells, JAG1 induces tumor cell growth and inhibits apoptosis. JAG1 also plays a key role in cancer stem cell maintenance and promotes metastasis. In the tumor microenvironment, JAG1 was also linked to promoting tumor angiogenesis and increasing the Treg cell population to inhibit tumor-specific immunity. The genes that are listed along the arrow leading to their corresponding role in cancer biology are regulated in a JAG1-dependent manner either in a direct or indirect manner. Genes represented in green are increased by JAG1 while the genes decreased by JAG1 are represented in red. DSL = Delta/Serrate/Lag-1, EGF = Epidermal Growth Factor, C2 = Calcium-binding, CRD = Cysteine-rich Domain, JICD = JAG1 Intracellular Domain, PDZ = PSD95/Dlg/ZO-1, ADAM = A-Disintegrin and Metalloproteinase, αvβ3-integrin = Integrin αv and Integrin β3, IL-2 = Interleukin-2, IL-6 = Interleukin-6, IL-10 = Interleukin-10, IL-17 = Interleukin-17, CD44 = Cluster of Differentiation 44, CD34 = Cluster of Differentiation 34, µPA = Urokinase-type Plasminogen Activator, MMP2 = Matrix Metallopeptidase 2, MMP9 = Matrix Metallopeptidase 9, DLL4 = Delta-like Ligand 4, Bcl-2 = B-cell Lymphoma-2, c-myc = V-myc Avian Myelocytomatosis Viral Oncogene Homolog, Foxp3 = Forkhead Box P3, PDGFR-β = Platelet-derived Growth Factor Receptor–β, RUNX3 = Runt Related Transcription Factor 3, SOX2 = Sex Determining Region Y (SRY) Box 2, ZEB1 = Zinc-finger E-box Binding Homeobox 1, OX40L = Tumor Necrosis Factor Receptor, Superfamily Member 4.

repeats) (Figure 10). The DSL family is classified in the JAG- or DLL-subgroups, but the defining motif that determines the JAG1-subgroup is the presence of a cysteine-rich domain (CRD) between the EGF-repeats and transmembrane domain (Luca, et al., 2017; Chillakuri, et al., 2013). The intracellular domains of DSL proteins vary in length and are not conserved in the primary amino acid sequence. Additionally, the JICD contains a PDZ-ligand binding domain located at the C-terminal end of the JAG1 receptor (Ascano, Beverly, & Capobianco, 2003; LaVoie & Selkoe, 2003) (Figure 10). The JICD is released after undergoing cleavage by ADAM and γ-secretase upon binding to the Notch receptor. Upon this release, the JICD induces intrinsic bidirectional signaling in the JAG1 expressing cell (Lu, et al., 2013) (Figure 8). Furthermore, JAG1-dependent Notch signaling is directly implicated in tumor growth by maintaining CSC populations (Simões, et al., 2015), promoting cell survival (Purow, et al., 2005), inhibiting apoptosis (Wang, et al., 2010a), and influencing cell proliferation (Cohen, et al., 2010) as well as metastasis (Leong, et al., 2007). Additionally, JAG1 can also affect cancer tumors indirectly by interacting with components of the tumor microenvironment to promote angiogenesis (Chen, et al., 2016) and T-cell regulation (Kumar, et al., 2017). Considering that the focus of this dissertation is based on the regulation of the Notch ligand receptor JAG1, this section will primarily focus on JAG1-dependent Notch signaling and its roles in cancer biology as well as its potential as a therapeutic target in breast cancer. Figure 10 depicts a synopsis of this section and contains an overview of the structure of JAG1 along with a summary of its roles in both direct and indirect regulation of gene expression for various functions in cancer biology.

2.3.1. Angiogenesis

Angiogenesis is the growth of new blood vessels from existing ones and is a physiological process that normally takes place during embryonic development and wound

healing (Carmeliet & Jain, 2011). Angiogenesis is also a hallmark function of cancer progression, as this process allows the tumor to receive nutrients (Hanahan & Weinberg, 2000). Notch ligands expressed on endothelial cells can interact with their respective receptors on endothelial and perivascular cells, smooth muscle cells, and pericytes, which are involved in multiple stages of blood vessel formation from initial sprouting until maturation of the blood vessel (Sainson & Harris, 2008). JAG1 has been proven to promote endothelial cell proliferation and sprouting and inhibit DLL4-dependent Notch signaling in endothelial cells (Kofler, et al., 2011). Therefore, inhibition of JAG1 reduces sprouting angiogenesis, while increased expression suppresses DLL4-dependent signaling to promote sprouting angiogenesis.

When it comes to vascular smooth muscle cell coverage of blood vessels and maintaining the interaction between endothelial cells and perivascular cells, JAG1 is indispensable. High et al. showed that the expression of JAG1 in endothelial cells is required for vascular smooth muscle development because its knockout from the endothelium induced defects in vascular smooth muscle turned out to be fatal (High, et al., 2008) due to JAG1-mediated increases in integrin αv and integrin β3 (αvβ3-integrin), which confers activity to von Willebrand Factor (VWF). The activation of VWF is important for maintaining smooth muscle adhesion and vessel maturation (Scheppke, et al., 2012). It was shown by Liu et al. that Notch3-expressing perivascular cells can also be induced by JAG1-expressing endothelial cells (Liu, Kennard, & Lilly, 2009). As a result, the upregulation of JAG1 expression on perivascular cells induces an auto-regulatory loop that promotes pericyte maturation and angiogenesis. This auto-regulatory loop is important, considering that inhibition of Notch signaling by Notch3 or JAG1 knockdown abrogates angiogenesis. The platelet-derived growth factor (PDGF) signaling pathway functions to promote angiogenesis when endothelial cells secrete the ligand platelet-derived growth factor-

β (PDGF-β) (Kofler, et al., 2011). Interestingly, the platelet-derived growth factor receptor-β (PDGFR-β) has been shown to be a direct target gene of JAG1-induced Notch activation (Liu, Kennard, & Lilly, 2009).

In the case of tumor associated blood vessels, JAG1 has been reported to be highly expressed in blood vessels that are in contact with brain and ovarian tumors (Lu, et al., 2007). In head and neck squamous cell carcinoma (HNSCC) tumors, growth factors such as human growth factor (HGF) and TGFα are secreted from the tumor and, in an autocrine and or juxtacrine fashion, upregulate the expression of JAG1 via the MAPK pathway. As a result, JAG1 then stimulates endothelial sprouting, promoting angiogenesis and tumor growth in the mouse model (Zeng, et al., 2005). Moreover, JAG1 expression has a positive correlation with micro-vessel formation in HNSCC. The pro-angiogenic role of JAG1 has also been reported in ovarian cancer models because silencing JAG1 expression in stromal cells significantly reduced tumor microvascular density and neoplastic growth (Steg, et al., 2011). Overall, this data suggests that inhibiting the JAG1 pathway could be beneficial to cancer patients, even in the absence of tumoral JAG1 expression due to its role in the promotion of angiogenesis.

2.3.2. Cancer Stem Cells

Even though CSCs were discussed extensively in the previous chapter, this section will cover the functionality of JAG1-dependent Notch signaling in the CSC population. Due to the significance of the Notch pathway in normal stem cell biology, treatment using GSIs in cancer patients to inhibit all Notch signaling factors (pan-Notch ablation) resulted in intolerable levels of GI toxicity (Imbimbo, 2008). Unlike the DLL1 and DLL4 Notch ligands, JAG1 was found to be dispensable for the homeostatic maintenance of normal intestinal stem cells (Pellegrinet, et al., 2011) suggesting that targeting JAG1 specifically is likely to have less GI side-effects.

Considering we know that Notch signaling is important for both CSC maintenance and self-renewal (Wang, Li, Banerjee, & Sarkar, 2009; Espinoza, Pochampally, Xing, Watabe, & Miele, 2013), several studies indicated that JAG1 is functionally linked to "stemness" in cancer and appears to be the primary ligand that drives Notch signaling in the CSC population (Patrawala, et al., 2005; Harrison, et al., 2010).

In breast cancer, it was shown that high levels of JAG1 promote mammosphere formation in vitro by mediating the pro-cancer effects of Notch3 (Sansone, et al., 2007a), interleukin-6 (IL-6) (Sansone, et al., 2007b), carbonic anhydrase IX (CAIX) (Lock, et al., 2013) and NF-κB (Yamamoto, et al., 2013). In a mouse model, deletion of lunatic fringe (Lfng), an N-acetylglucosamine transferase that prevents JAG1-dependent Notch activation, induced basal-like breast cancer with higher JAG1 activity and enhanced the CSC proliferation (Xu, et al., 2012). JAG1 is also involved in CSC biology in other tumor types such as glioblastoma (Zhu, et al., 2011), lymphoma (Cao, et al., 2014), and colorectal cancer (Lu, et al., 2013). Overall, these observations indicate that JAG1 is a vital inducer of the CSC phenotype in different types of cancer, which suggests its targeting could suppress CSCs.

2.3.3. Epithelial-Mesenchymal Transition (EMT), Invasion, and Metastasis

Metastasis is a complex process with multiple steps that gives tumor cells the ability to invade the surrounding tissue and to colonize distant organs; both are key features of cancer aggressiveness. To escape their local environment, epithelial cells can exploit a reversible developmental process called the epithelial-mesenchymal transition (EMT) (Hanahan & Weinberg, 2011). EMT is a phenotypic switch that converts epithelial cells into motile mesenchymal-like cells, which requires epithelial cells to undergo a spectrum of changes that reduce their adhesion to other cells and increase their migratory process. EMT is essential during

embryonic development and in tissue repair (Kong, Li, Wang, & Sarkar, 2011), but a large body of evidence indicates that it contributes to the early stages of tumor metastasis. Notch signaling has been extensively studied in this context and several reports describe JAG1 involvement in EMT, invasion, and metastasis in breast cancer and other cancer types (Espinoza, Pochampally, Xing, Watabe, & Miele, 2013). Leong et al. demonstrated that JAG1-induced Notch signaling in breast cancer could inhibit the epithelial phenotype by increasing expression of EMT master-regulator SLUG, which in turn promoted tumor growth and metastasis (Leong, et al., 2007). JAG1 was also shown to be integrated with TGFβ-induced EMT in mammary gland cells (Zavadil, Cermak, Soto-Nieves, & Böttinger, 2004). Moreover, JAG1 increases the migratory and invasive behavior of TNBC cells through the induction of urokinase-type plasminogen activator (μPA), a well-established marker of recurrence and metastasis (Shimizu, et al., 2011). Lastly, JAG1 has been identified to be involved in the tissue specificity of breast cancer dissemination, as was described to play a vital role in metastasis to the bone and brain (Sethi, Dai, Winter, & Kang, 2011; Xing, et al., 2013).

JAG1 was also described to promote EMT in other cancer types such as 1) cervical cancer, where JAG1 expression positively correlated with induction of PI3K-mediated EMT (Veeraraghavalu, et al., 2005), in 2) hepatocellular carcinoma, where JAG1 was reported to suppress the expression of tumor suppressor runt related transcription factor 3 (RUNX3) (Tanaka, et al., 2012), and in 3) drug resistant pancreatic cancer, where JAG1-Notch2 signaling promoted several EMT transcription factors such as Snail, Slug, and zinc-finger E-box binding homeobox 1 (ZEB1) (Wang, et al., 2009). In prostate cancer, JAG1 was able to promote invasive and migratory functions through the NF-κB signaling pathway (Wang, et al., 2010a). Lastly, in colon cancer, JAG1 mediated apurinic/apyrimidinic endodeoxyribonuclease 1 (APEX1) pro-

tumorigenic functions and induced expression of metastatic markers matrix metallopeptidase 2 (MMP2) and matrix metallopeptidase 9 (MMP9) (Kim, et al., 2013; Dai, et al., 2014). As a whole, JAG1-dependent Notch signaling appears to be implicated in various steps of EMT, invasion, and metastasis, which was demonstrated in several tumor types and further promotes the interest of JAG1 as a therapeutic target to inhibit tumor cell invasiveness and metastasis.

2.3.4. Proliferation, Cell Cycle Regulation, Drug Resistance, and Survival

JAG1 regulates basic cellular functions such as cell cycle progression, proliferation, apoptosis, and cell survival. In several types of cancer, JAG1 was shown to induce tumor cell growth and to promote cell cycle progression. For example, Cohen et al. showed that JAG1 can directly regulate the cell cycle by identifying CCND1 as a direct target of JAG1-dependent Notch activation in breast cancer cells (Cohen, et al., 2010). The same observation was also demonstrated in colon cancer where JAG1-dependent Notch signaling induced the expression of CCND1, cyclin E (CCNE), and c-myc (Dai, et al., 2014). In prostate cancer, JAG1 promoted cyclin-dependent kinase 2 (CDK2) activity while repressing p27, a cell cycle suppressor (Zhang, et al., 2006). Similar proliferative promoting functionality has also been reported in glioma (Purow, et al., 2005), myeloma (Jundt, et al., 2004), and ovarian cancer (Choi, et al., 2008; Steg, et al., 2011). Knowing the role of JAG1 in cell proliferation, it is not surprising that JAG1 is indicated as the Notch ligand that exerts anti-apoptotic functions. Evidence for the anti-apoptotic functions of JAG1 was produced by Wang et al. who demonstrated that JAG1 suppressed apoptosis by activating serine-threonine protein kinase (Akt), mechanistic target of rapamycin (mTOR), and NF-κB signaling pathways in glioma cells and by upregulating Bcl-2 (Wang, et al., 2010a). In regard to drug resistance, JAG1 prevented chemotherapy-induced apoptosis in lymphoma with respect to doxorubicin (Cao, et al., 2014), taxanes with ovarian cancer (Steg, et

al., 2011), breast cancer with tamoxifen (Simões, et al., 2015), and pancreatic cancer with gemcitabine (Wang, et al., 2009). The chemo-protective effects of JAG1 represent a key finding as it highlights the opportunity to treat drug resistant cancers via combination therapy that includes inhibition of JAG1-dependent Notch signaling through the potential use of monoclonal antibodies or small molecules.

2.3.5. T-cell Regulation

The tumor microenvironment is a vital component for cancer growth and survival. There is a plethora of immune cells that are found within close proximity of tumors that include T-cells, DCs, macrophages and neutrophils. However, there are very few natural killer cells present, which are extremely effective in killing tumor cells (Whiteside, 2008). It is important to note that in many cancer types, there is an increased concentration of T-regulatory (Treg) cells that can suppress immune responses that are tumor-specific, which in turn allows tumor cells to evade tumor surveillance (Strauss, et al., 2007). Treg cells in combination with myeloid-derived suppressor cells and cytokines help foster and maintain a favorable immunosuppressive tumor microenvironment that supports tumor growth (Whiteside, 2008).

JAG1-induced Notch activation plays an important role in the induction and expansion of Treg cells in the tumor microenvironment, which involves cross-talk between tumor cells and DCs. JAG1-Notch3 signaling has been identified to stimulate tumor necrosis factor superfamily receptor 4 (OX40L) in order to initiate Treg cell induction and expansion (Gopisetty, et al., 2013), while JAG1 expression in antigen presenting cells induces antigen-specific Treg cells. Furthermore, the maturation of DCs via JAG1 is what promotes the survival and proliferation of Treg cells (Bugeon, Gardner, Rose, Gentle, & Dallman, 2008). JAG1-induced Notch activation may also have an impact on CD46 T-cell activation, which was demonstrated by Le Friec et al.

by identifying a new interaction between JAG1 and CD46 (Le Friec, et al., 2012). CD46 is a ubiquitously expressed human type I transmembrane glycoprotein that is currently identified as a complement regulatory protein and cell-entry receptor (Le Friec, et al., 2012). The activation of CD46 on $CD4^+$ T-cells was shown to regulate the expression of Notch as well as its ligands. Furthermore, JAG1 was identified as an additional physiological ligand for CD46, as the JAG1 binding site of CD46 overlaps with that of Notch receptors. Therefore, targeting JAG1-dependent Notch signaling may promote the eradication of tumor cells by augmenting the effects of cancer chemotherapeutics and by bolstering the capability of the immune system to destroy cancer cells.

2.3.6. A Synopsis of JAG1-Notch Signaling in Breast Cancer

To date, there are multiple lines of evidence that establish Notch signaling in the involvement of breast cancer development, maintenance and metastasis (Takebe, Nguyen, & Yang, 2014). Over expression of Notch1, Notch3, and Notch4-activated ICDs caused aggressive and metastatic mammary tumors in mice (Kofler, et al., 2011; Simões, et al., 2015), while active forms of Notch1 and Notch4 receptors induce transformation of both normal human and murine mammary epithelial cells (Leong, et al., 2007). Additionally, loss of Numb, a negative regulator of Notch signaling, is found to be associated with higher grade and poorer prognosis in breast cancer (Chen, et al., 2016), while suppression of Notch activity via Notch3 and CSL knockdown promotes cancer cell apoptosis and inhibits tumor cell growth (Liu, Kennard, & Lilly, 2009).

It is important to note that unlike in T-cell acute lymphoblastic leukemia (T-ALL), which normally stems from Notch1 gene mutations (Vlierberghe & Ferrando, 2012), the induction of Notch signaling in breast cancer, as well as others, is mainly associated with ligand-dependent methods of activation. Studies currently indicate that JAG1 is the most prominent ligand

involved in excessive activation of Notch signaling in breast cancer as both messenger RNA (mRNA) and protein are over expressed in breast cancer and these higher expressions are established indicators of poor prognosis (Dickson, et al., 2007; Reedijk, et al., 2005; Buckley, et al., 2013). Therefore, it is critical to identify methods and or natural cellular mechanisms that inhibit or reduce JAG1 expression as a means for the treatment and prevention of various cancers.

CHAPTER 3

THE ARYL HYDROCARBON RECEPTOR (AHR): A MULTI-FACETED TRANSCRIPTION FACTOR

3.1. A Brief History and Introduction to AHR Biology

During the Vietnam War, US and allied military sprayed approximately 77 million liters of tactical herbicide (Yi & Ohrr, 2014). Agent Orange was the most commonly used agent and was contaminated with 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD). In animals, TCDD is a lipophilic environmental toxicant that acts as a potent multisite carcinogen and has been shown to disrupt multiple endocrine pathways (Vanden Heuvel & Lucier, 1993; Sycheva, et al., 2016; Rappe, 1990). Later, it was discovered that TCDD binds its cognate cytoplasmic ligand-activated transcription factor, the aryl hydrocarbon receptor (AHR). TCDD is very stable and highly selective for the AHR, making it the ideal ligand for discovering AHR-regulated genes and pathways. To date, most TCDD effects have been reported to be mediated via the AHR and changes in gene expression. Although TCDD is best known for inducing developmental and immune toxicity, there are many reports showing that it also modulates several cancer processes (Warner, et al., 2011) including cell invasion (Hall, et al., 2010), movement (Zhang, et al., 2012), and proliferation (Zhang, et al., 2009).

Since the Vietnam War and over the past 50 years, there have been advances in understanding the role of the AHR in both cancerous and normal tissue. The industrial revolution during the twentieth century promoted extremely rapid growth in the mass production of various plastic and metal materials. As a result, increased chemical waste production led to increased risk of exposure to potentially hazardous environmental pollutants/toxicants. Chemical spills and accidental exposure to polycyclic halogenated aromatic hydrocarbons (PHAHs) or polycyclic

aromatic hydrocarbons (PAHs) have unveiled a wide array of detrimental health effects, which has been and is continuing to be extensively studied by researchers (Zack & Suskind, 1980; Collins, Bodner, Aylward, Wilken, & Bodnar, 2009). In 1949, one of the earliest PAH/PHAH chemical spills occurred in Nitro WV, about 40 miles from Marshall University, and industrial workers that manufactured Agent Orange were exposed to the AHR agonist TCDD. Aside from being a carcinogen, exposure to TCDD resulted in chloracne, liver disease, ischemic heart disease, and even death in some workers (Collins, Bodner, Aylward, Wilken, & Bodnar, 2009). However, the exact mechanism by which TCDD causes toxicity in humans is still unclear.

The discovery of benzpyrene hydroxylase was a result of some of the earliest research with PAHs (Conney, Miller, & Miller, 1957). Benzpyrene hydroxylase was an enzyme named due to its ability to form hydroxylated products of 3,4-benzpyrene, but it was quickly renamed aryl hydrocarbon hydroxylase (AHH) because several PAHs induced its activity (Nebert & Bausserman, 1971; Gielen & Nebert, 1971). During the 1970s, there were numerous studies showing that there was a direct correlation between activated AHH and increased cytochrome P450, family 1 subfamily A member 1 (CYP1A1) activity; and that this response varied between different mouse strains, specifically C57BL/6 and DBA/2 mice (Nebert & Bausserman, 1970). 3-Methylcholanthrene (3-MC) was able to stimulate increases in AHH activity in C57BL/6 mice more effectively than DBA/2 mice. Researchers wondered whether diminished AHH activity in DBA/2 mice in response to 3-MC was due to these mice having a genetic defect, or whether they express a PAH/PHAH receptor with reduced binding affinity (Poland, Glover, Robinson, & Nebert, 1974). Researchers used radioactive TCDD to discover that it accumulated highly in the liver of C57BL/6 mice, where it bound a receptor leading to induction of AHH activity (Poland, Glover, & Kende, 1976). In DBA/2 mice, the levels of radio-labeled TCDD in liver extracts were

much lower, supporting the previous theory that they express a receptor with reduced binding affinity. These radioactive TCDD experiments allowed the Nebert lab to dub this newly identified receptor, the AHR (Okey, et al., 1979). During this time, it was also determined that the AHH and CYP1A1 were one and the same; CYP1A1 will be used henceforth to refer to this important enzyme (Okey, 2007).

The Bradfield group validated the hypothesis that DBA/2 mice expressed an altered AHR that was proposed in the 1970s when they cloned the AHR in 1992. These studies revealed that reduced AHR ligand affinity was due to an altered AHR ligand binding domain in DBA/2 mice (Burbach, Poland, & Bradfield, 1992). When the AHR coding sequences from the DBA/2 and C57BL/6 were compared, two critical alterations were noted: 1) the AHR had undergone a substitution (valine instead of alanine) at position 375 and 2) a T to C mutation in the stop codon was observed in the DBA/2 mouse strain (Ema, et al., 1994). The mutated stop codon causes a lengthening of the carboxy-terminus in the mature AHR protein in DBA/2 mice resulting in a ligand-binding domain with lowered ligand binding affinity (Ema, et al., 1994). The major advances in AHR biology, after these initial findings were discovered, was made possible by uncovering the canonical AHR signaling pathway and will be discussed in the next section.

3.2. The Canonical AHR Signaling Pathway

When the AHR is activated by a ligand such as TCDD, it becomes a transcription factor by moving into the nucleus to regulate the expression of various genes (Figure 11). In the cytoplasm, the inactive AHR is bound with two HSP90 proteins (Chen, Singh, & Perdew, 1997) (Figure 11). These HSP90 proteins function as chaperones to prevent degradation of the AHR as well as for steroid hormone receptors (Pratt & Toft, 1997) (Figure 11). The inactivated AHR complex also contains a third protein, which was identified as hepatitis B Virus X-associated

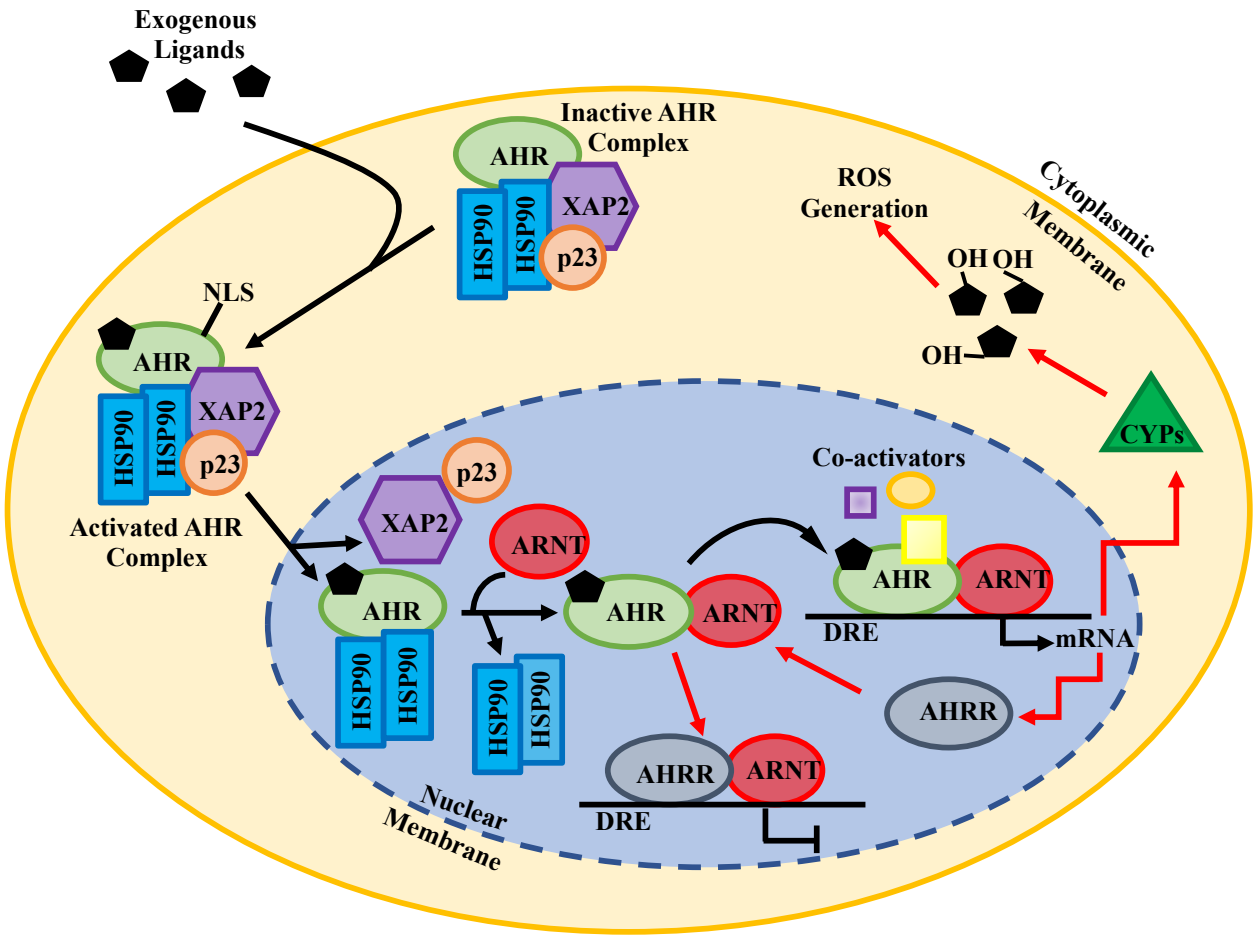


Figure 11. The Canonical AHR Signaling Pathway
Ligands diffuse through the cellular membrane and bind to the cytosolic AHR complex, which in turn exposes the NLS. The activated AHR complex is then translocated into the nucleus where the ARNT displaces chaperone proteins and binds the AHR, resulting in AHR-ARNT dimers that bind to and activate transcription from gene promoters that contain DREs such as various CYP450s. Activation of CYP450s can result in metabolism of exogenous and endogenous AHR ligands. The AHRR lacks a transactivation domain and exerts negative-feedback regulation on the AHR pathway through its competition for ARNT proteins and formation of inactive AHRR-ARNT transcriptional complexes on DREs. Following transcription, AHR is exported and degraded by the proteasome in the cytoplasm. NLS = Nuclear Localization Sequence, AHRR = Aryl Hydrocarbon Receptor Repressor, AHR = Aryl Hydrocarbon Receptor, XAP2 = Hepatitis B Virus X-associated Protein 2, HSP90 = Heat-shock Protein 90, ARNT = Aryl Hydrocarbon Receptor Nuclear Translocator, DRE = Dioxin Response Element, OH = hydroxyl group, CYP450s = Cytochrome P450s. Red Arrows = Post-activation.

protein 2 (XAP2) (Carver & Bradfield, 1997; Meyer, Pray-Grant, Vanden Heuvel, & Perdew, 1998). However, the function of XAP2 has not been determined. Later research showed that p23 was also part of the inactive AHR complex in the cytoplasm, and it was identified to help maintain AHR stability (Figure 11). This inactive AHR complex serves to keep the AHR from entering the nucleus by concealing the AHR nuclear localization sequence (NLS) in the absence of ligand (Petrulis, Hord, & Perdew, 2000; Kazlauskas, Poellinger, & Pongratz, 1999; Petrulis, Kusnadi, Ramadoss, Hollingshead, & Perdew, 2003). Once TCDD binds to the AHR, a conformational change occurs in the complex that exposes the NLS of the AHR and translocates the activated complex into the nucleus (Jain, Dolwick, Schmidt, & Bradfield, 1994) (Figure 11). Once the complex enters the nucleus, XAP2 and p23 are released and the AHR binds to the ARNT with its bHLH motif (Rowlands, McEwan, & Gustafsson, 1996) (Figure 11). As a result, the ARNT keeps the AHR in the nucleus and induces the release of the HSP90 chaperones, leading to the activation of gene expression via the TCDD-AHR-ARNT complex (Figure 11).

Canonical AHR signaling stimulates the expression of genes involved in phase I and phase II drug metabolism, such as CYP1A1 and cytochrome P450, family 1 subfamily B member 1 (CYP1B1) (Korashy, Abuohashish, & Maayah, 2013). In the liver, these monooxygenase enzymes are important for "first pass" metabolism where a large portion of drugs and xenobiotics are bio-transformed via hydroxylation in order to aid in the excretion and elimination of various compounds by increasing their solubility in water (Meyer, 1996). Cytochrome P450s 1A1 and 1B1 have been highly associated with cancer. CYP1A1 has been shown to regulate breast cancer proliferation and survival as well as initiate cancer growth by converting toxicants such as benzo[α]pyrene (B[α]P) into genotoxic agents (Rodriguez & Potter, 2013; Schwarz, Kisselev, Cascorbi, Schunck, & Roots, 2001). Silencing of CYP1A1 resulted in

decreased colony formation, proliferation, and CCND1 levels as well as inducing cell cycle arrest and apoptosis in human breast cancer cells (Rodriguez & Potter, 2013). CYP1B1 has been coined as a "universal tumor marker" due to its over-expression in many tumor types, including lung, breast, and colorectal tumors (Murray, et al., 1997). CYP1B1 has been shown to convert estrogen into genotoxic metabolites like 4-hydroxyestradiol to initiate tumor formation. Glutathione-S-transferases (GSTs) are phase II enzymes whose expression is also induced by TCDD via AHR activation (Li, Seidel, Pritchard, Wolf, & Friedberg, 2000; Schrenk, Stüven, Gohl, Viebahn, & Bock, 1995). GSTs aid in the detoxification of xenobiotics by catalyzing the conjugation with GSH (Ketterer, Coles, & Meyer, 1983). Specific polymorphisms in the genes that encode the GST enzyme superfamily have been reported to be linked with resistance to chemotherapy in tumors (McIlwain, Townsend, & Tew, 2006).

The AHR and ARNT target genes possess a unique sequence in a specific region of DNA called dioxin response elements (DREs). The specific consensus DNA sequence for the TCDD-AHR binding complex (5'-TGCGTGA-3') was first identified in the CYP1A1 gene promoter, which lies upstream and proximal to the transcription start site (TSS). The function of the DREs is to confer transcriptional activation to TCDD-AHR target genes upon binding TCDD-stimulated AHR-ARNT complexes (Denison, Fisher, & Whitlock, 1988). In addition to AHR-ARNT, TCDD also stimulates the recruitment of transcriptional cofactors to DREs in TCDD-AHR target genes including acetyltransferase p300 (Beischlag, Luis Morales, Hollingshead, & Perdew, 2008; Beischlag, et al., 2002), CBP (Bedford & Brindle, 2012), nuclear receptor co-activator 1 (NCOA-1) (Spencer, et al., 1997) and brahma-related gene 1 (BRG1) (Trotter & Archer, 2008). These transcriptional cofactors stimulate gene expression by promoting histone

acetylation and inducing chromatin to assume a confirmation that is more conducive to active transcription (Spencer, et al., 1997).

The discovery of DREs was a landmark moment in AHR biology and gave way for the identification of other genes regulated by TCDD, such as ATP binding cassette sub-family G member 2 (ABCG2) (Tan, et al., 2010) and aldehyde dehydrogenase 1, family member A3 (ALDH1A3) (Marcato, et al., 2011; Contador-Troca, et al., 2015). The transporter ABCG2 promotes the efflux of drugs from tumor cells while ALDH1A3 is overexpressed in BCSCs and has been identified as a prognostic marker (Marcato, et al., 2011). The transcriptional regulation of CYP1A1 (Rodriguez & Potter, 2013), CYP1B1 (Murray, et al., 1997), ABCG2 (Tan, et al., 2010), ALDH1A3 (Marcato, et al., 2011) has been shown to play roles in several different types of cancer, indicating that a better understanding of AHR signaling could provide new insights into the modulation of several cancers including breast cancer. One of the objectives in this dissertation was to use genome-wide RNA-sequencing (RNA-seq) analysis to identify novel TCDD-AHR regulated genes that could play important roles in the regulation of breast cancer progression. My findings revealed that stimulating human MCF7 breast cancer cells with TCDD for 6 hours induced changes in the expression of 660 genes including: CYP1A1, CYP1B1, ABCG2 and ALDH1A3. This novel result indicates that TCDD-AHR signaling may modulate breast cancer initiation and progression by inducing changes in the expression of a large set of genes that regulate various cancer processes. Indeed, my RNA-seq results provided the impetus to investigate a novel hypothesis in this dissertation by which TCDD-AHR signaling modulates breast cancer progression by regulating the JAG1-Notch1 pathway. As detailed in chapters 1 and 2, the JAG1-Notch signaling pathway is an embryonic pathway that also promotes breast cancer progression.

3.3. Non-canonical AHR Signaling

Although the canonical AHR signaling pathway is important, AHR activation can also alter gene expression by cross-talking with other important transcription factors. The AHR has also been shown to cross-talk with the ER (Matthews & Gustafsson, 2006), NF-κB (Denison, Soshilov, He, DeGroot, & Zhao, 2011; Vogel, et al., 2014), and HIF-1α (Vorrink & Domann, 2014) to modulate estrogen, inflammatory and hypoxia signaling, respectively.

There are various ways that the AHR can cross-talk with transcription factors, but the interaction between the AHR and ER has been the most extensively studied (Figure 12). Experiments studying long-term TCDD exposure in female Sprague Dawley rats were the first to suggest that the AHR and ER signaling pathways cross-talk, because it was identified that TCDD inhibited the growth of estrogen responsive mammary and uterine tumors (Kociba, et al., 1978). Other reports have shown that TCDD inhibited estrogen-stimulated increases in the expression of ER target genes (Safe & Wormke, 2003). There are several mechanisms by which TCDD-AHR signaling influences estrogen signaling (Figure 12). The first mechanism is mediated by CYP1A1-induced metabolism of estrogen upon exposure to TCDD. As noted earlier, CYP1A1 reduces estrogen by promoting its metabolism (Spink, et al., 1992) (Figure 12). Other recent findings have identified that TCDD-activated AHR signaling stimulates increases in ER ubiquitination and degradation (Wormke, et al., 2003) as well as inhibits estrogen-stimulated gene expression by binding to inhibitory DREs (iDREs) in the promoter regions of ER target genes (Krishnan, Porter, Santostefano, Wang, & Safe, 1995) (Figure 12). Because the AHR and ER share common transcriptional co-activators, they have been reported to compete for a limited pool of transcriptional co-activators, which can lead to reductions in ER transcriptional activity

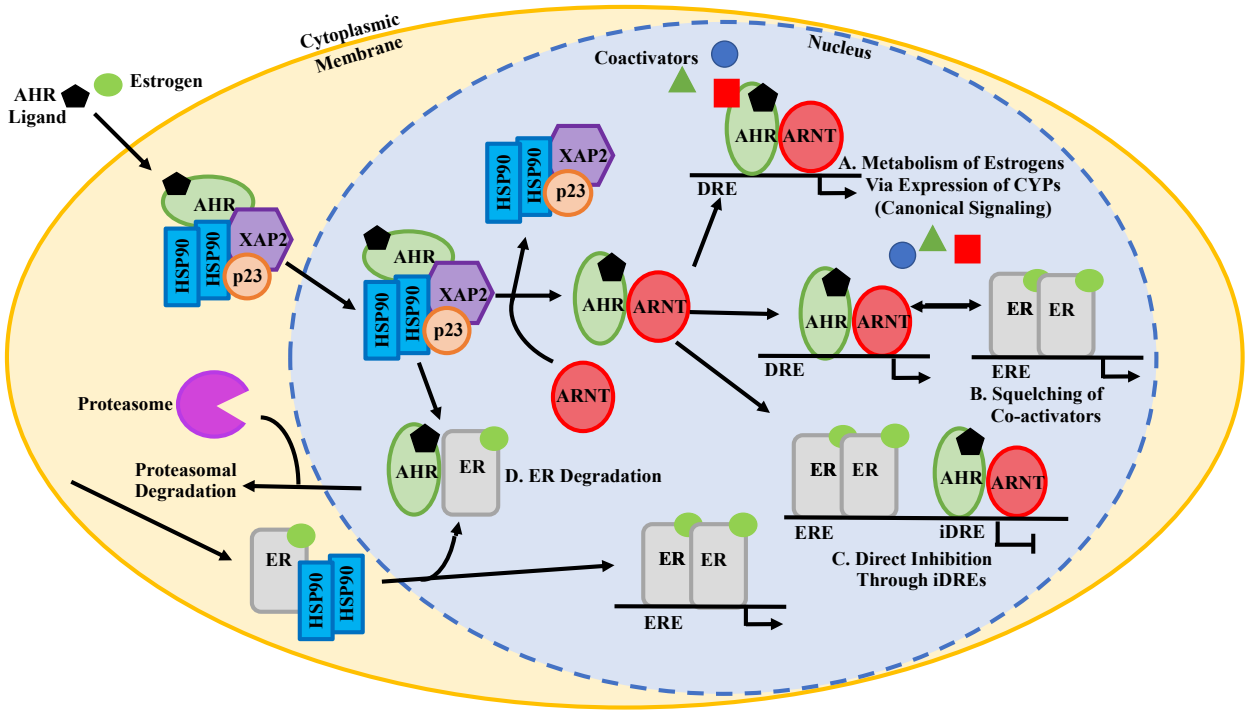


Figure 12. Mechanisms of AHR-ER Crosstalk
(A) AHR activation upregulates CYP450 expression and promotes estrogen metabolism. (B) Activation of AHR squelches co-activator proteins preventing them from binding with ER transcriptional complexes. (C) Activated AHR-ARNT complexes can bind to iDREs in ER target genes to silence expression. (D) The AHR targets ER for proteasomal degradation. ER = Estrogen Receptor, AHR = Aryl Hydrocarbon Receptor, ARNT = Aryl Hydrocarbon Receptor Nuclear Translocator, DRE = Dioxin Response Element, ERE = Estrogen Response Element, iDRE = Inhibitory Dioxin Response Element, HSP90 = Heat Shock Protein 90, XAP2 = Hepatitis B Virus X-associated Protein 2.

(Brunnberg, et al., 2003) (Figure 12). TCDD has also been reported to mimic estrogen signaling by promoting the recruitment of unliganded ER to the estrogen response element (ERE) in ER target genes (Brunnberg, et al., 2003).

TCDD has also been reported to crosstalk with the NF-κB pathway (Tian, 2009; Beischlag, Luis Morales, Hollingshead, & Perdew, 2008; Kim, et al., 2000; Salisbury & Sulentic, 2015). The NF-κB canonical pathway is induced by pro-inflammatory cytokines, IL-6 and TNFα (Yamamoto, et al., 2013; Hayden & Ghosh, 2008; Kolasa, Houlbert, Balaguer, & Fardel, 2013) (Figure 13). IL-6, by activating its cognate receptor, induces the phosphorylation of inhibitor of NF-κB (IκB) by the inhibitor of NF-κB kinase (IκBK) complex, leading to its ubiquitination and subsequent degradation by the 26S proteasome (Hayden & Ghosh, 2008) (Figure 13). The V-Rel avian reticuloendotheliosis viral oncogene homolog A (RelA)-p50 complex is then free to translocate to the nucleus to activate the transcription of target genes (Hayden & Ghosh, 2008; Bash, et al., 1999) (Figure 13). TNFα induces a non-conical NF-κB pathway, which results in the activation of IκB by NF-κB Inducing Kinase (NIK) (Yamamoto, et al., 2013) (Figure 13). The formation of the NIK-IκB-p100 complex leads to the phosphorylation of the p100 subunit (Hayden & Ghosh, 2008) (Figure 13) and results in 26S proteasome-dependent processing of p100 to p52. The production of p52 leads to the activation of the V-Rel avian reticuloendotheliosis viral oncogene homolog B (RelB)-p52 heterodimer that targets a distinct NF-κB element and induces the transcription of target genes (Vogel & Matsumura, 2009) (Figure 13). The AHR crosstalks with the NF-κB pathway by binding to RelA and forms an inactivated AHR-RelA dimer that perturbs RelA-stimulated transcription (Salisbury & Sulentic, 2015; Tian, 2009) (Figure 13). The AHR has also been reported to bind to RelB, forming an active AHR-RelB heterodimer that increases the transcriptional activity of AHR-RelB response

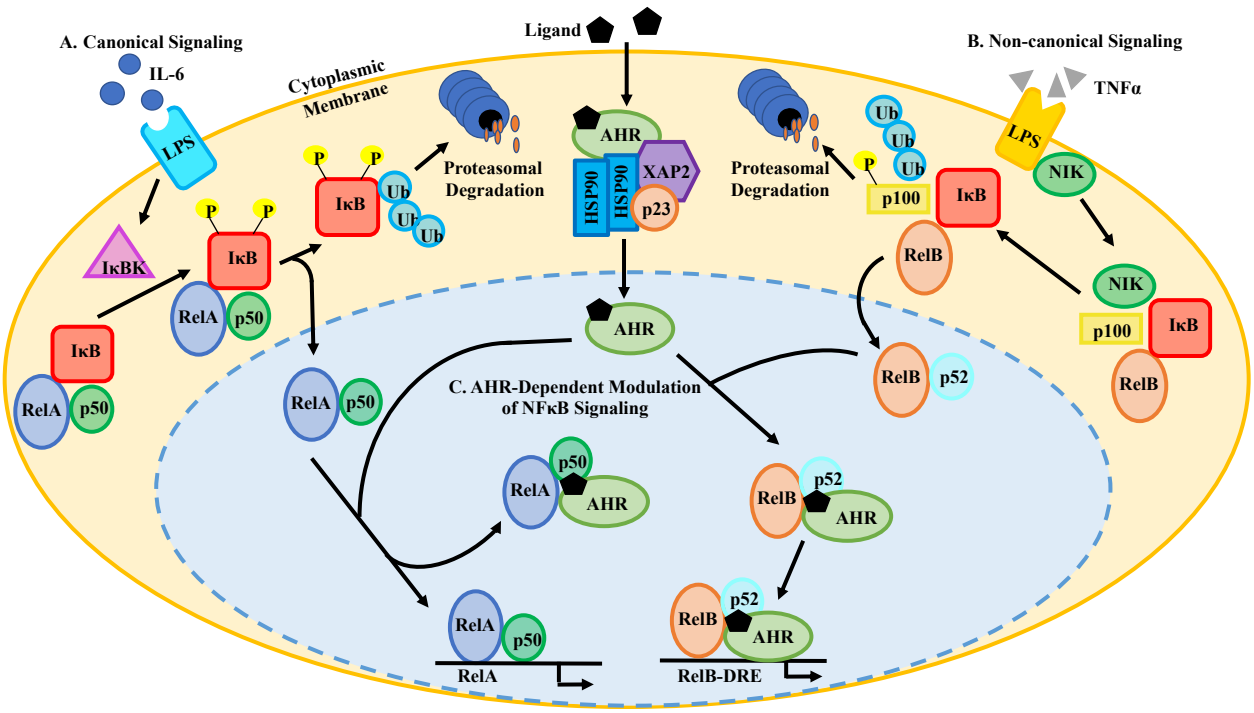


Figure 13. Canonical and Non-canonical Pathways of NF-κB Signaling Modulated by AHR
A) The canonical pathway is induced by cytokines such as IL-6. The activation results in the phosphorylation of IκB by the IκBK complex, leading to its ubiquitylation and subsequent degradation by the 26S proteasome. The RelA-p50 complex is free to translocate to the nucleus to activate the transcription of target genes. B) The non-canonical pathway results in the activation of IκBK by the NIK after stimulation by cytokines. The formation of the NIK-IκB-p100 complex leads to the phosphorylation of the p100 subunit and results in 26S proteasome dependent processing of p100 to p52 and leads to the activation of the RelB-p52 complex that target distinct NF-κB elements and induce the transcription of target genes. C) Direct protein binding between the AHR and RelA was proposed to induce repressive effects that are mediated by formation of transcriptionally inactive AHR-RelA heterodimers that also reduced the concentration of available nuclear AHR and RelA necessary for normal AHR- and NF-κB-mediated gene expression. In contrast to RelA, direct interactions between the TCDD-activated AHR and RelB also occur and were reported to produce an active AhR-RelB heterodimer complex that could bind to a specific AHR-RelB DNA recognition site and stimulate transcription of inflammatory genes. AHR = Aryl Hydrocarbon Receptor, XAP2 = Hepatitis B Virus X-associated Protein 2, HSP90 = Heat-shock Protein 90, RelA = V-Rel Avian Reticuloendotheliosis Viral Oncogene Homolog A, RelB = V-Rel Avian Reticuloendotheliosis Viral Oncogene Homolog B, IκB = Inhibitor of NFκB, IκBK = Inhibitor of NF-κB Kinase, Ub = ubiquitin, NIK = Nuclear Factor κB-inducing Kinase, TNFα = Tumor Necrosis Factor α, IL-6 = Interleukin-6, LPS = Lipopolysaccharide, p = phosphate group.

elements in specific pro-inflammatory genes such as interleukin 8 (IL-8) (Vogel & Matsumura, 2009; Vogel, et al., 2014; Vogel, et al., 2007) (Figure 13).

The AHR has also been seen to cross-talk with HIF-1α (Vorrink & Domann, 2014) (Figure 14). Oxygen is essential for aerobic organisms and is an important component of many cellular processes such as aerobic metabolism and energy homeostasis (Majmundar, Wong, & Simon, 2010). The amount of available oxygen in tissues can become reduced under certain physiological conditions or disease states. The key players that regulate the adaptive responses to hypoxia are the hypoxia-inducible factors. HIF-1α, one of the main regulators of the hypoxic response, is a transcription factor that also requires the ARNT protein as a dimerization partner (Kewley, Whitelaw, & Chapman-Smith, 2004). Under normal oxygen (normoxic) conditions, HIF-1α undergoes hydroxylation via prolyl hydroxylase domain (PHD) proteins and then subsequently ubiquinated by the von Hippel-Lindau tumor suppressor gene product (pVHL) before undergoing proteasomal degradation (Déry, Michaud, & Richard, 2005; Ohh, et al., 2000) (Figure 14). However, hypoxic conditions stabilize HIF-1α, and this transcription factor induces gene expression by forming a heterodimer with the ARNT (Kewley, Whitelaw, & Chapman-Smith, 2004) (Figure 14). In cancer, the activation of HIF-1α-ARNT signaling can maintain and promote the self-renewal capacity of GSCs by promoting the Notch signaling pathway (Qiang, et al., 2012). The high binding-affinity of the ligand-activated AHR to ARNT proteins compete against HIF-1α for a limited pool of ARNT proteins and other co-activators, which in turn can negatively impact the activity of HIF-1α (Vorrink & Domann, 2014) (Figure 14).

3.4. TCDD-induced Negative-feedback Loops that Limit AHR Signaling

There are two TCDD-induced negative-feedback loops that limit AHR signaling (Figure 15). Soon after its induction by TCDD, the AHR is ubiquitinated and then degraded by the

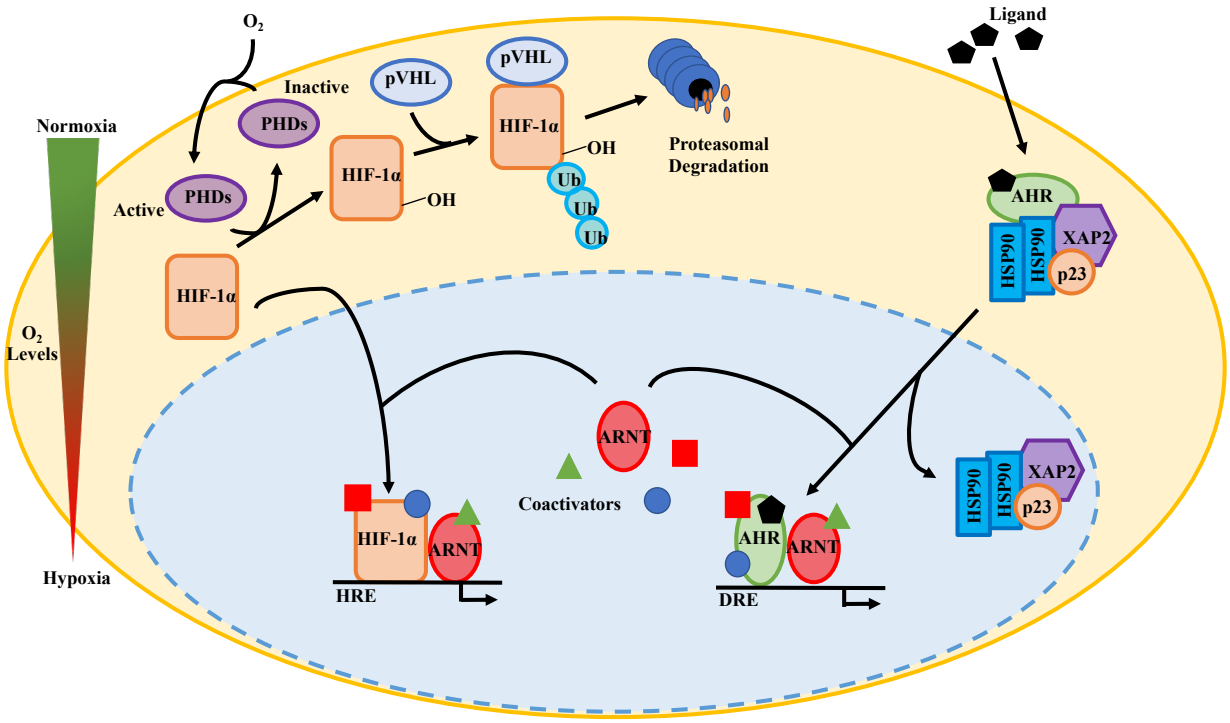


Figure 14. Crosstalk at the AHR-ARNT-HIF-1α Signaling Node
Normoxic conditions lead to HIF-1α hydroxylation and degradation. HIF-1α proteins are hydroxylated by PHD proteins, ubiquinated by pVHL and ultimately degraded via the proteasome. In contrast, hypoxic environments cause HIF-1α stabilization and translocation to the nucleus. In the nucleus, HIF-1α binds to the ARNT to induce transcription of HRE-containing genes. The AHR is quiescent in the cytoplasm until ligand binding occurs, which initiates translocation to the nucleus. In the nucleus, the AHR binds to the ARNT to induce transcription of DRE-containing genes. AHR = Aryl Hydrocarbon Receptor, XAP2 = Hepatitis B Virus X-associated Protein 2, HSP90 = Heat-shock Protein 90, ARNT = Aryl Hydrocarbon Receptor Nuclear Translocator, DRE = Dioxin Response Element, HRE = Hypoxic Response Element, HIF-1α = Hypoxia Inducible Factor 1α, PHD = Prolyl Hydroxylase Domain, pVHL = von Hippel-Lindau Tumor Suppressor Gene Product, OH = Hydroxyl Group, Ub = Ubiquitin, O_2 = Oxygen.

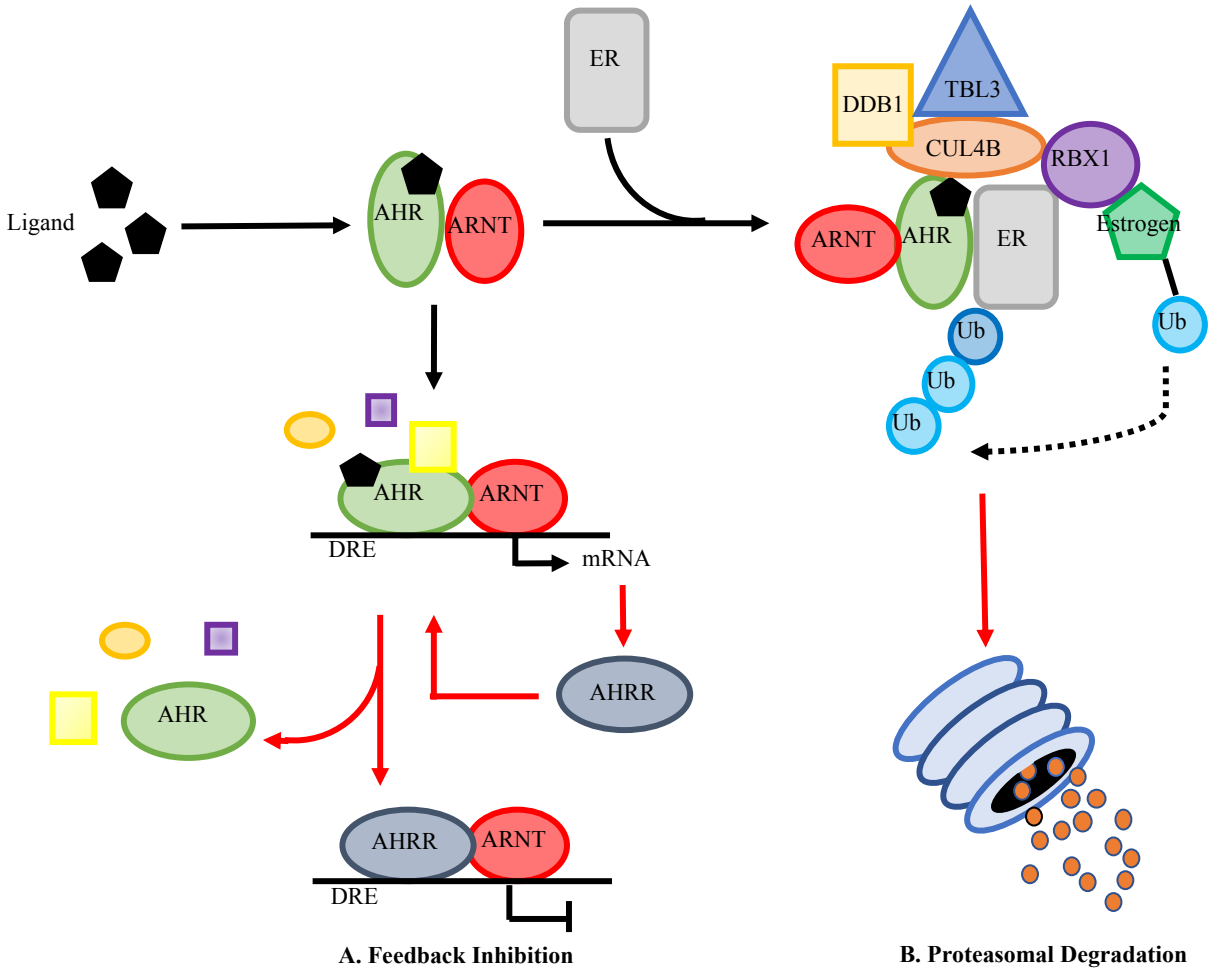


Figure 15. Mechanisms of Inhibition for AHR
(A) The activated AHR binds the ARNT and co-activators to regulate expression of target genes containing DREs and induce expression of the AHRR. The AHRR protein competes with ARNT proteins for binding the AHR, which prevents active AHR complex formation and binding to DREs to regulate gene expression. (B) AHR signaling can also be inhibited through proteasomal degradation via CUL4B and other ubiquinating enzymes. AHR = Aryl Hydrocarbon Receptor, ARNT = Aryl Hydrocarbon Nuclear Translocator, RBX1 = Ring Box 1, DDB1 = Damaged DNA Binding Protein 1, Ub = Ubiquitin, CUL4B = Cullin 4B, ER = Estrogen Receptor, TBL3 = Transducin Beta Like 3, DRE = Dioxin Response Element, AHRR = AHR Repressor. Red Arrows = Post-activation.

proteasome (Ma & Baldwin, 2000) (Figure 15). The proteins that mediate the ubiquitination of TCDD-induced AHR include: cullin 4B (CUL4B), ring box 1 (RBX1) and damaged DNA binding protein 1 (DDB1), which are recruited by AHR in response to TCDD (Morales & Perdew, 2007) (Figure 15). Interestingly, TCDD-induced AHR also promotes the ubiquitination of the ER and the AR, which in part explains how TCDD inhibits estrogen and androgen signaling (Ohtake, Fujii-Kuriyama, & Kato, 2009) (Figure 15). The second TCDD-induced negative-feedback loop that limits AHR signaling involves the AHR repressor (AHRR) (Figure 15).

The AHRR and AHR contain a bHLH motif and, therefore, AHRR proteins can bind to ARNT proteins (Evans, et al., 2008) (Figure 15). TCDD induces the transcription and expression of the AHRR, which in turn removes ARNT proteins from the AHR and suppresses AHR activity (Mimura, Ema, Sogawa, & Fujii-Kuriyama, 1999) (Figure 15). However, other reports suggest that AHRR binds directly to DREs in AHR target genes, which also inhibits AHR activity (Hahn, Allan, & Sherr, 2009) (Figure 15). Thus, the primary mechanism by which TCDD-induced AHRR exerts its negative feedback inhibition on AHR signaling is still not clear; however, it is clear that AHRR suppresses TCDD-induced AHR signaling.

3.5. Physiological Roles of AHR: Immunity, Development, and Reproduction

For reasons detailed in the previous paragraphs, the historical role of AHR has been linked with TCDD and TCDD-induced toxicity. However, the development of AHR-null mice during the 1990s showed that the AHR has significant activity in mice not treated with TCDD. Indeed, AHR-null mice exhibited defects in immunity, proper organ development, and reproduction. For instance, AHR-null mice showed decreased lymphocyte accumulation in the spleen and lymph nodes compared to wild-type mice, but not the thymus (Fernandez-Salguero,

Hilbert, Rudikoff, Ward, & Gonzalez, 1996; Schmidt, Su, Reddy, Simon, & Bradfield, 1996; Andreola, et al., 1997). This suggested that AHR signaling has a role in innate immunity. Further investigation into the role of the AHR in immunity has identified that different AHR ligands have different effects on T-cells. For instance, TCDD and the endogenous AHR ligand kynurenine promote the activity and differentiation of T-cells into Treg cells, which in turn suppresses immune responses and favors the growth of tumors (Mezrich, et al., 2010; Quintana, et al., 2008). However, the endogenous AHR ligand 6-formylindolo [3,2-b] carbazole (FICZ) stimulates the differentiation of naïve T-cells into pro-inflammatory T-helper 17 (Th17) cells and this exacerbates immune responses (Veldhoen, et al., 2008). Some dietary AHR ligands such as indolo [3,2-b] carbazole (ICZ) and 3,3-diindolylmethane (DIM) have also been shown to play a role in the immune response (Veldhoen & Brucklacher-Waldert, 2012). Indole-3-carbinol (I3C) is a dietary AHR ligand that comes from cruciferous vegetables and is converted by stomach acid to the products ICZ and DIM, which then act as high affinity AHR ligands in the gut. As a result, ICZ- and DIM-activated AHR helps maintain gut microflora, intraepithelial lymphocytes, interleukin-22 (IL-22) expression and Th17 cell activity (Veldhoen & Brucklacher-Waldert, 2012). These studies and observations allowed researchers to establish that there are AHR-dependent roles in developmental and functional immunity.

The liver expresses the highest levels of the AHR compared with all other tissues (Dolwick, Schmidt, Carver, Swanson, & Bradfield, 1993). The liver of AHR-null mice is smaller and exhibits thickening, and fibrosis of the portal vein compared with wild-type mice, suggesting that proper liver development is AHR-dependent (Schmidt, Su, Reddy, Simon, & Bradfield, 1996). Further work has fostered a hypothesis by which the observed diminished liver growth in AHR-null mice is attributed to significant inflammatory changes in the bile ducts (cholangitis)

compared to wild-type mice (Fernandez-Salguero, et al., 1995). Although the activation of the AHR by endogenous ligands is important for proper development of the liver, its activation by TCDD causes developmental and endocrine toxicity. For instance, TCDD dramatically reduced ovarian follicle number and size in female Holtzman rats, which provided a mechanism that explains why TCDD reduces fecundity in female rats (Heimler, Trewin, Chaffin, Rawlins, & Hutz, 1998). In a rodent model, other teratogenic effects include blocked ovulation (Li, Johnson, & Rozman, 1995), atypical release of FSH and LH (Li, Johnson, & Rozman, 1997) as well as development of cleft palate and kidney deficiencies in offspring (Mimura, et al., 1997). Work comparing wild-type mice with AHR-null mice has confirmed that TCDD-induced toxicity is mediated through AHR signaling. Mouse studies have revealed that AHR has a role in mammary development and function, including pregnancy-associated changes in the mouse mammary gland and in utero mammary gland development. More specifically, AHR-null mice exhibit improper mammary development compared to wild-type mice, including reductions in mammary gland size and less mammary gland terminal end buds, which are the proliferative structures found at the tips of ducts (Mimura, et al., 1997). This result suggests that normal development and proliferation in the mammary gland could be dependent on endogenous AHR ligands.

3.6. AHR Signaling and Cancer

There have been numerous reports linking AHR signaling to cancer biology and a high degree of complexity has emerged with pro-tumorigenic and anti-tumorigenic activities of the AHR in studies using cell culture and in vivo models of malignancy (Murray, Patterson, & Perdew, 2014). Whether the AHR promotes or inhibits the initiation or progression of cancer is dependent on the ligand it binds. Consequently, there are tumor promoting AHR ligands as well as tumor suppressive AHR ligands. The activity of these ligands are also cancer specific. The

AHR modulates these tumor-promoting and -suppressing pathways in different cancer types and this seemingly indecisive role of AHR in cancer has been attributed to 1) differences in cancer models, 2) species differences, 3) different AHR ligands having different (and opposite) effects in cancer and 4) that AHR exhibits promiscuous binding with multiple ligands that have different chemical structures (Denison, Soshilov, He, DeGroot, & Zhao, 2011). The importance of defining whether the AHR is tumor promoting or tumor suppressing in each cancer type has important implications for drug development, because AHR antagonism may work well for some cancers, and AHR agonism may work better for other cancers. The following section will provide a balanced discussion of the tumor-promoting and -suppressing roles for the AHR in cancer. Although the AHR has been proposed to bind hundreds of ligands, I will focus my discussion on the best characterized AHR ligands in cancer, which are shown in Table 3 and 4.

There are multiple cases and studies identifying AHR signaling promotes tumorigenic activity. Overexpression of the AHR and constitutively active AHR signaling is common in breast and prostate cancer, as well as other cancers (Shin, Sánchez-Velar, Sherr, & Sonenshein, 2006; Kolluri, Jin, & Safe, 2017; Schlezinger, et al., 2006). Moreover, the AHRR has also been identified as a tumor suppressor (Zudaire, et al., 2008). Overexpression of the AHR in normal human mammary cells induces malignant traits such as increased EMT (Barretina, et al., 2012; Brooks & Eltom, 2011), a cellular process where cells lose adhesion to each other in preparation to invade and or migrate. Endogenous AHR ligands that are products of tryptophan metabolism such as kynurenine and kynurenic acid have also been identified to promote tumor survival by suppressing the immune response through autocrine and paracrine mechanisms (Opitz, et al., 2011; DiNatale, et al., 2010; D'Amato, et al., 2015). CYP1B1, a known AHR gene target, has also been identified to be highly expressed in metastatic tumors compared to healthy tissue and

AHR Agonists that Possess Anti-cancer Effects			
Source	**AHR Agonists**	**Cell Lines that Exhibited Anti-Cancer Effects**	
Xenobiotic	**Polycyclic Halogenated Aromatic Hydrocarbons (PHAHs):** • TCDD	• LNCaP • MCF7 • SKBR3	• MDA-MB-231 • BT474 • T47D
	Pharmaceuticals: • Tranilast	• Panc-1 • MiaPaCa2 • MCF7	• MDA-MB-231
	• Omeprazole	• Panc-1 • MiaPaCa2 • MCF7	• MDA-MB-231
Dietary	**Flavonoids:** • Quercetin	• MCF7 • HL60	• CCRF-CEM
	• Kaempferol	• MCF7 • PaCa-2	• Panc-1
	Indoles: • Indole-3-carbinol (I3C)	• MCF7 • MDA-MB-231	• MDA-MB-468
	• 3,3'-Diindoylmethane (DIM)	• MCF7 • MDA-MB-231	• SKBR3 • T47D
Endogenous	**Tryptophan Metabolites**: • 2-(1'H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methylester (ITE)	• HCCLM3 • OVVAR-3 • SKOV-3	• HepG2 • LNCaP • U87

Table 3. AHR Agonists that Possess Anti-cancer Effects

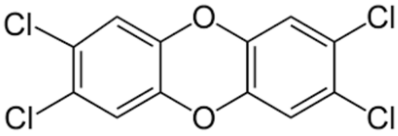
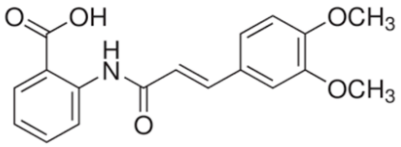
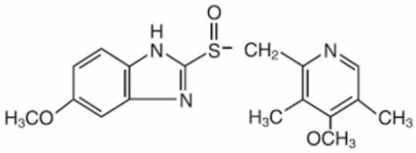
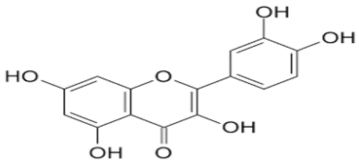
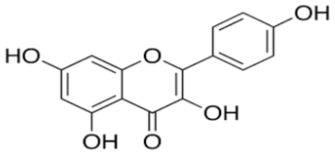
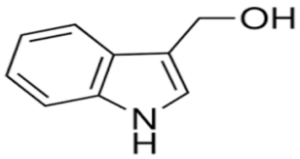
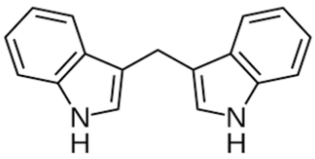
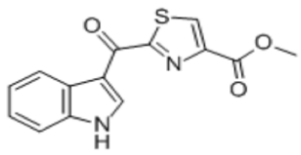
Molecular Structures of AHR agonists that Promote Anti-cancer Effects	
AHR Agonist	**Molecular Structure**
TCDD	
Tranilast	
Omeprazole	
Quercetin	
Kaempferol	
Indole-3-carbinol	
3,3’-Diindolylmethane	
2-(1’H-indole-3’-carbonyl)-thiazole-4-carboxylic acid methylester (ITE)	

Table 4. The Molecular Structures of AHR Agonists that Promote Anti-cancer Effects

has been identified as a metastatic marker in lung, colon, prostate, and breast cancer (Murray, et al., 1997; Murray, Melvin, Greenlee, & Burke, 2001). Elevated CYP1B1 levels promote the synthesis of the genotoxic metabolite 4-hydroxyestradiol and increases chemo-resistance by metabolizing certain chemotherapy agents into inactive metabolites (Li, Seidel, Pritchard, Wolf, & Friedberg, 2000).

TCDD is considered as both a complete epigenetic carcinogen and a potent tumor promoter through sustained AHR activation (Safe, 1990; Poland & Knutson, 1982). However, whether TCDD is a tumor promoter or a tumor suppressor is dependent on the cancer. For instance, TCDD inhibits the proliferation of ER-positive breast cancer cells, because it inhibits estrogen signaling (Hanieh, 2015; Wang, Wyrick, Meadows, Wills, & Vorderstrasse, 2011). TCDD has also been reported to inhibit the proliferative, migratory and invasive activity of breast cancer cells that are of the TNBC subtype (Zhang, et al., 2009; Hall, et al., 2010). The application of TCDD to TNBC cells induced decreases in the expression of sex-determining region Y (SRY)-box 4 (SOX4), which is a transcription factor that promotes metastatic behavior of breast cancer cells (Hanieh, 2015). TCDD suppressed the expression of SOX4 by inducing the expression of the microRNA-212/132 cluster, which directly targets SOX4 (Hanieh, 2015). Although TCDD is too toxic to be used for cancer treatments, finding that TCDD inhibits the aggressiveness of TNBC cells suggests that increasing the activity of AHR with a non-toxic AHR ligand could be a new way to treat this disease.

With regard to non-toxic AHR ligands (Tables 3 and 4), dosing rodents with the dietary AHR ligand I3C inhibited the growth of mammary (Wu, et al., 2010; Tin, Park, Sundar, & Firestone, 2014) and cervical (Qi, Anderson, Chen, Sun, & Auborn, 2005) tumor xenografts without causing toxicity. In addition to mammary and cervical cancer, I3C has also been reported

to suppress prostate cancer progression in vivo (rodent models) by promoting cell cycle arrest and increasing apoptosis (Chinni & Sarkar, 2002). The search for endogenous AHR ligands led to the purification and characterization of an endogenous ligand with high affinity and selectively for AHR, known as 2-(1'H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methylester (ITE) (Song, et al., 2002; Henry, Bemis, Henry, Kende, & Gasiewicz, 2006). ITE was purified from porcine lung. Recent reports indicate that ITE has anticancer activity that is attributed to its function as an AHR ligand (Wang, et al., 2013). The application of ITE to glioma cancer cells inhibited the expression of octamer-binding protein 4 (OCT4), which encodes a transcription factor that plays a key role in embryonic development and stem cell pluripotency (Cheng, et al., 2015). Upregulation of OCT4 in adult tissues promotes tumorigenesis. Therefore, finding that ITE inhibited OCT4 provided a new insight into how this endogenous AHR ligand inhibited the stem cell-like cancer cells in glioblastoma. Importantly, in contrast to TCDD, ITE is not toxic when tested in rodent models (Nugent, et al., 2013). The dosing of mice with ITE, however, suppressed the growth of glioblastoma tumor grafts in nude mice, indicating that this endogenous AHR ligand has anti-cancer activity in vivo (Cheng, et al., 2015).

The anti-inflammatory drug tranilast functions as an AHR ligand and it has been reported to inhibit mammary cancer in mice (Rogosnitzky, Danks, & Kardash, 2012). Interestingly, tranilast inhibits the BCSC population in vivo and in vitro models at pharmacologically relevant concentrations (200 micromolar (μM)) (Prud'homme, et al., 2010). Further work showed that knockdown of AHR prevented the effects of tranilast, indicating that this anti-inflammatory drug inhibits breast cancer by activating AHR (Prud'homme, et al., 2010). The translation of AHR ligands for breast cancer therapy into the clinic mandates the identification of non-toxic AHR ligands that have anti-cancer activity in vivo and a complete understanding of the pathways that

are regulated by AHR ligands. In this regard, my work in this dissertation has identified for the first time that the non-toxic AHR ligand ITE inhibits TNBC cell aggressiveness by reducing the levels of JAG1 and NICD, which are known to promote breast cancer progression.

3.7. Current Problematic Observation and Overall Hypothesis

Although there are various AHR ligands that have been shown to promote various anti-cancer effects, the mechanism(s) by which they occur have yet to be determined or fully characterized. As a result, the controversy of utilizing non-toxic AHR ligands as a potential therapy along with current chemotherapy regimens is still prominent, unless a more concrete mechanism behind the anti-cancer effects is proposed. Characterizing the mechanism that promotes these anti-cancer effects via non-toxic AHR ligands may also shed light on specific proteins or signaling pathways that may also be considered to be novel therapy targets for the treatment of cancer. It is also important to note that investigating the changes in JAG1 levels in the presence of known anti-cancer AHR ligands can provide important insight on how the expression of JAG1 in cancer cells as well as changes in cell migration and invasion are altered in a ligand-dependent manner in cancer cells (Hall, et al., 2010). Therefore, I hypothesize that non-toxic AHR ligands can induce anti-cancer effects through regulating the expression of JAG1 and the Notch signaling pathway.

CHAPTER 4

METHODS FOR STUDYING AHR SIGNALING

4.1. Materials

The purpose of this introductory methods section is to provide a brief overview of the methods that were used in this dissertation. MCF7 (ER-positive), T47D (ER-positive) MDA-MB-231 (ER-negative) breast cancer cells were purchased from ATCC (Manassas, VA). MDA-MB-157 (ER-negative) cells were generously provided by Dr. Ruth Keri (Case Western Reserve). TCDD in dimethyl sulfoxide (DMSO) (155μM) was purchased from Cambridge Isotopes Laboratory (Andover, MA). ER status of these cell lines were determined via western blot. The AHR agonist ITE was purchased from Tocris Bioscience (San Diego, CA). The TCDD antagonist CH-223191 was purchased from Sigma-Aldrich (St. Louis, MO). Non-targeting siRNA (cat # D-001810-01-20), ON-TARGET plus human siRNA against AHR (cat # J-004990-08-0010) and JAG1 (cat # J-011060-11-0005) were purchased from GE Dharmacon (Lafayette, CO). Dulbecco's Modified Eagle Medium (DMEM)/High glucose with L-glutamine and sodium pyruvate, fetal bovine serum (FBS), phosphate buffer saline (PBS), penicillin/streptomycin, and DMSO were purchased from Thermo Fisher Scientific (Pittsburgh, PA). Sodium dodecyl sulfate (SDS), 30% acrylamide/bis solution, ammonium persulfate, Tween-20, β-mercaptoethanol (BME) and polyvinylidene difluoride (PVDF) membranes were obtained from BIO-RAD (Hercules, CA).

4.2. RNA-sequencing

RNA-sequencing (RNA-seq) analysis was based on four biological replicates in each experimental group. For TCDD stimulation, MCF7 (ER-positive) cells were plated at 250,000 cells/milliliter (mL) on 35 millimeter (mm) cell culture plates in DMEM containing 10% FBS

(DMEM/FBS (10%)) and were grown for 24 hours before being serum starved in phenol red-free DMEM for 16 hours prior to treatment. Cells were then treated with TCDD (100 nanomolar (nM)) for 6 hours. Total RNA purification kits (Qiagen, Valencia, CA) were used to extract total RNA. RNA sample quality was assessed using Bioanalyzer RNA Nano chips (Agilent); all RNA samples had an RNA Integrity Number (RIN) greater than or equal to 8. The RIN is an algorithm for assigning integrity values to RNA measurements and assigns an electropherogram a value of 1 to 10, with 10 being the least degraded. RNA-seq libraries were prepared from 1 microgram (μg) of total RNA using a TruSeq RNA Prep Kit (Illumina Inc., San Diego, CA). RNA-seq was performed using an Illumina HiSeq1000 in a 2 x 100 base paired end design yielding a minimum of 50 million reads per sample. Differentially expressed genes were identified at a False Discovery Rate (FDR) of 5%. For pathway analysis, TCDD-regulated genes (TRGs) expressed as a ratio of TCDD/DMSO were loaded into Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, Redwood City, CA). The IPA Core Analysis tool was used to identify significant associations between TRGs and pathways. We configured the core analysis to report Benjamini-Hochberg corrected p-values.

4.3. Cell Culture and Treatments

Cell lines were maintained at 37°C with 5% carbon dioxide (CO_2). Cells were grown in DMEM/FBS (10%) and 1% pencillin/streptomyocin. Cells were plated onto 35mm tissue culture plates in DMEM/FBS (10%) at a cell density of 250,000 cells per plate 24 hours prior to treatment. The medium containing ITE (10μM) was reapplied to cells every 12 hours, based on the findings of a prior report showing that ITE induces its maximal effect at 10μM, and it has to be applied to cells every 12 hours based on its stability in cell culture (Cheng, et al., 2015; Henry, Bemis, Henry, Kende, & Gasiewicz, 2006). The medium containing TCDD (10nM or

100nM) was applied once to cells, because TCDD is stable in cell culture. The AHR antagonist CH-223191 (10μM) was applied 1 hour prior to treatment with ITE or TCDD.

4.4. Short-interfering RNA (siRNA): Gene Knockdown Experiments

Gene specific siRNA were used to knockdown the expression of specific genes to evaluate their function. A concentration of 200,000 cells/mL in DMEM/FBS (10%) were mixed directly with 100nM of siRNA that was either non-targeting (control), JAG1-targeting or AHR-targeting. Transfection reagent (3 microliters (μL)) (Lipofectamine RNAi Max, Life Technologies) was then added and plated onto 35mm tissue culture plates. After 36 hours, cells were treated with vehicle, TCDD (10nM, 24 hours), or ITE (10uM, 72 hours). Treatments were removed, and total cellular RNA or protein was extracted with RNA-purification kits (Qiagen RNeasy) or 2× Laemmli sample lysis buffer, respectively. In some experiments, total RNA was isolated using TRI-reagent in accordance with protocols provided by the manufacturer (Sigma Aldrich).

4.5. mRNA Analysis: Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNA was extracted using RNA purification columns (Qiagen). Reverse transcription was performed on 300 nanograms (ng) of RNA using cDNA synthesis kits (Applied Biosystems (Foster City, CA)) in accordance with the suppliers' instructions. StepOnePlus (Applied Biosystems) was used to take quantitative polymerase chain reaction (qPCR) measurements. Amplifications were carried out using SYBR green master mixes (Applied Biosystems) according to the manufacturer's protocols. Samples were run in triplicate, and the average normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was quantitated using the $2^{-\Delta\Delta CT}$ formula. CYP1A1 and GAPDH primers have been published (Tomblin & Salisbury, 2014). JAG1 primers used were: forward 5′-GAGCCCGGCCTCCTTTTATT, reverse

5′-GCGTCATTGTGTTACCTGCG.

4.6. Protein: Western Blotting

Cells were scraped in 300uL of 2x SDS sample buffer to isolate total cellular extract. Proteins were heat denatured and then separated by SDS/polyacrylamide gel electrophoresis (PAGE) followed by transfer to a PVDF membrane. Equal protein loading (~15µg/sample) was confirmed by western blotting. Blots were incubated overnight at 4°C in primary antibody followed by a 1 hour incubation in secondary antibody at room temperature. Antibodies from Cell Signaling Technology were: JAG1 (IC4) (1:1000), AHR (D5S6H) (1:1000), Cleaved-NICD (D3B8) (1:1000), HES1 (D6P2U) (1:1000), Snail (C15D3) (1:1000), rabbit-HRP (1:10,000) and mouse-HRP (1:10,000). GAPDH antibody was purchased from Millipore (Cat #MAB374). Densitometry was calculated with ImageLab PC-based software (Biorad).

4.7. Functional Assay: Scratch Analysis

Scratch assays were conducted with MDA-MB-231 cells. Cells were first seeded into 12-well plates at a concentration of 50,000 cells per well and were grown for 24 hours in DMEM/FBS (10%) and 1% pencillin/streptomyocin. Cells were then rinsed with PBS solution and treated with vehicle or 10µM ITE every 12 hours for 5 days in DMEM containing 1% FBS (DMEM/FBS (1%)). After 5 days, a scratch was made in each well with a pipette tip. The media was then aspirated to remove floating cells, then vehicle or 10µM ITE treated complete media was reapplied. The scratches were photographed at 0 hour and at 24 hours using the Lycia Microscope. The exposed surface area of the plates was measured using ImageJ analysis software. Regarding ITE (10µM) treatments, cells were grown and treated in the same manner as detailed in section 4.3, except ITE treatments, which continued for five days before the scratch

was made; ITE treatments were reapplied every 12 hours for five days. As for JAG1 siRNA transfections, JAG1 knockdown cells were scratched and incubated for 12 hours; cells were photographed at 0 hours and 24 hours.

4.8. Functional Assay: Boyden Chamber

Cells were plated on 35mm tissue culture plates in DMEM/FBS (10%) and 1% penicillin/streptomycin for 24 hours prior to treatment. After cells were treated with vehicle or 10μM ITE in DMEM/FBS (10%) and 1% penicillin/streptomycin every 12 hours for the indicated number of days, they were detached from tissue culture plates (using trypsin) and counted. Cells were immediately transferred to cell invasion chambers in DMEM at a concentration of 50,000 cells/well. Chambers filled with cells were then incubated in 24-well tissue culture plates containing DMEM/FBS (10%) as the chemoattractant for 36 hours. Each experiment included a negative control in which chambers filled with cells were incubated with DMEM lacking FBS. Following the 36h incubation in the presence of DMSO vehicle or 10μM ITE, a cotton swab removed non-adherent cells that were not invasive. Invasive cells were stained with crystal violet for 10 minutes at room temperature and then rinsed gently with tap water. Stained cells were then incubated in DMSO for 10 minutes with orbital shaking to extract crystal violet. Cell lysates were measured at 560 nanometers (nm) for invasive activity. For the indicated siRNA experiments, the cell invasion was assayed 48 hours after transfection. Boyden cell invasion chambers were purchased from ThermoFisher Scientific.

4.9. Statistical Analysis

The Tukey's post-hoc test was used to determine statistically significant differences among groups following one-way analysis of variance (ANOVA). One-tailed, unpaired t-tests with confidence intervals of 95% were used to determine statistically significant differences

between two groups. All studies were conducted in triplicate.

CHAPTER 5

ARYL HYDROCARBON RECEPTOR LIGANDS REDUCE JAGGED1 EXPRESSION IN BREAST CANCER CELLS

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5.1. Abstract

The AHR is a ligand-activated transcription factor that binds pollutants, therapeutic drugs, and endogenous ligands. The AHR is expressed in all breast cancer subtypes and it can switch the aggressiveness of breast cancer cells from low to high depending on the ligand that it binds. JAG1 is a Notch receptor ligand that is overexpressed in TNBC and promotes breast cancer progression in part by increasing the migratory and invasive activity of TNBC cells. The regulation of JAG1 by the AHR in ER-positive and ER-negative breast cancer cells by two AHR ligands (TCDD and ITE) was investigated in this report in order to determine the anti-metastatic roles of AHR activation. TCDD is the prototype AHR ligand, and ITE is a non-toxic endogenous AHR ligand with anti-cancer activity. IPA revealed a significant association between TRGs and cell movement. Experiments involving siRNA-directed knockdown of AHR confirmed TCDD-stimulated decreases in JAG1 required AHR expression. TCDD-induced reductions in JAG1 were also inhibited by the AHR antagonist CH-223191. The non-toxic AHR ligand ITE also reduced JAG1 by activating the AHR in breast cancer cells, as confirmed by AHR knockdown experiments. RNA-seq findings coupled with published TCDD-stimulated AHR-chromatin immunoprecipitation (ChIP)-sequencing (ChIP-seq) results suggest that ligand-activated AHR reduces the expression of JAG1 by increasing the expression of HES1. HES1 is a direct AHR target gene and is also known as a JAG1 suppressor. MDA-MB-231 cells are TNBC cells that are highly migratory and invasive, and these cancer cell attributes were significantly inhibited by ITE. We reduced JAG1 with targeting siRNA, and the outcome mirrored ITE by suppressing TNBC cell migration and invasive activity. Collectively, these findings are the first showing that ITE is a tumor-suppressing AHR ligand in TNBC cells in part because it reduces JAG1 expression.

5.2. Introduction

The AHR mediates tumor-promoting and -suppressing pathways in different cancer types (Safe, Lee, & Jin, 2013; Murray, Patterson, & Perdew, 2014). The seemingly indecisive role of the AHR in cancer, sometimes tumor-promoting, other times tumor-suppressing, has been attributed to differences in cancer models, species differences and the receptor's promiscuous binding with multiple ligands that have different chemical structures including: halogenated polycyclic hydrocarbons (i.e. TCDD), polycyclic aromatic hydrocarbons (i.e. benzo[a]pyrene), tryptophan-derived ligands (i.e. kynurenine) and indole glucosinolates (i.e. indole-3-carbinol) (Denison, Soshilov, He, DeGroot, & Zhao, 2011; Murray, Patterson, & Perdew, 2014). The industrial byproduct TCDD is the prototype AHR ligand, whose combined stability and selectivity for the AHR exceeds all other AHR ligands (Denison, Soshilov, He, DeGroot, & Zhao, 2011). Gene expression changes induced by TCDD, therefore, reflect the purest AHR response that can be obtained by RNA-seq. The AHR is a cytoplasmic protein that binds HSP90 (Perdew, 1988; Kazlauskas, Sundstrom, Poellinger, & Pongratz, 2001), XAP2 (Carver & Bradfield, 1997; Meyer, Pray-Grant, Vanden Heuvel, & Perdew, 1998) and the co-chaperone protein p23 (Kazlauskas, Poellinger, & Pongratz, 1999). The binding of TCDD to the AHR induces AHR translocation from the cytoplasm to the nucleus where it forms a heterodimer with the ARNT (Denison, Soshilov, He, DeGroot, & Zhao, 2011; Beischlag, Luis Morales, Hollingshead, & Perdew, 2008). Primary TCDD-stimulated AHR target genes harbor inducible DREs that confer transcriptional activity to TCDD target genes upon their binding to AHR activated complexes (Beischlag, Luis Morales, Hollingshead, & Perdew, 2008).

TNBC cells fail to express ER, PR and HER2 (Foulkes, Smith, & Reis-Filho, 2010). Absence of these three receptors make TNBC more difficult to treat, because there are no

targeting therapies for this breast cancer subtype (Foulkes, Smith, & Reis-Filho, 2010). The AHR, however, is expressed in all TNBC cells studied to date (Zhang, Liu, Wang, & Zhang, 2016; Tomblin, et al., 2016; Hall, et al., 2010). Further, TCDD signaling reduces the aggressiveness of TNBC cells by suppressing their proliferation (Zhang, Liu, Wang, & Zhang, 2016), promoting their differentiation (Hall, et al., 2010) and suppressing their invasive activity (Zhang, et al., 2012). This is contrary to the AHR ligand kynurenine where it is known to promote the survival of TNBC cells by suppressing anoikis pathways (D'Amato, et al., 2015). Thus, AHR may increase or decrease the aggressiveness of TNBC cells depending on which ligand it binds. The effects of ITE, a nontoxic endogenous AHR ligand, (Henry, Bemis, Henry, Kende, & Gasiewicz, 2006; Song, et al., 2002) has not been investigated in breast cancer cells. However, the known anti-cancer activity of ITE in other types of cancer (Cheng, et al., 2015; Wang, et al., 2013) motivated us to test its effects on breast cancer cells.

The Notch signaling pathway has also been implicated in breast cancer progression. There are five Notch receptor ligands (JAG1, JAG2 and DLL1, DLL3, and DLL4); however, JAG1 is the predominant tumor-promoting Notch receptor ligand in breast cancer (Li, Masiero, Banham, & Harris, 2014; Previs, Coleman, Harris, & Sood, 2015). Upon binding JAG1, the Notch receptor undergoes two proteolytic cleavage events (Borggrefe & Liefke, 2012) that release the NICD (Bray, 2006). Following its release, the NICD translocates to the nucleus and binds to the transcription factor RBP-Jκ (Borggrefe & Oswald, 2009), which induces the exchange of corepressors for coactivators at RBP-Jκ and this in turn stimulates increases in the transcription of NICD gene targets (Bray, 2006).

In this report, we performed an RNA-seq analysis to identify TRGs in MCF-7 breast cancer cells and identified 660 TRGs. HES1 was identified as a direct AHR target gene that

functions as a transcriptional repressor and a regulator of neurogenesis. HES1 is also a downstream target gene of the Notch signaling pathway and has been identified to suppress the expression of Notch receptor ligands, JAG1 and DLL1, as a negative-feedback regulatory mechanism. Interestingly, JAG1 which is associated with breast cancer recurrence and poor breast cancer outcome (Reedijk, et al., 2005; Dickson, et al., 2007; Buckley, et al., 2013), was one of the 660 TRGs. Because JAG1 is associated with poor breast cancer outcomes (Buckley, et al., 2013; Li, Masiero, Banham, & Harris, 2014), we investigated its regulation by two AHR ligands, TCDD and ITE, in order to determine if the anti-cancer effects of AHR activation was due to regulating JAG1 expression. Our results are the first to show that TCDD and ITE reduce JAG1 expression in breast cancer cells and confirm that JAG1 is critical for TNBC cell migratory and invasive activity.

5.3. Materials and Methods

5.3.1 Materials

MCF7 (ER-positive), T47D (ER-positive) MDA-MB-231 (ER-negative) breast cancer cells were purchased from ATCC (Manassas, VA). MDA-MB-157 (ER-negative) were generously donated by Ruth Keri (Case Western University). TCDD in DMSO was purchased from Cambridge Isotopes Laboratory (Andover, MA). The AHR antagonist CH-223191 was purchased from Sigma-Aldrich (St. Louis, MO). The AHR ligand ITE was purchased from Tocris Bioscience (Bristol, United Kingdom). Non-targeting siRNA (D-001810-01-20), ON-TARGET plus siRNA against AHR (J-004990-08-0010) and JAG1 (J-011060-11-0005) were purchased from GE Dharmacon (Lafayette, CO). DMEM/High glucose with L-glutamine and sodium pyruvate, FBS, PBS, penicillin/streptomycin, DMSO, and Lipofectamine RNAiMAX were purchased from Fisher Scientific (Pittsburgh, PA). SDS, 30% acrylamide/bis solution,

ammonium persulfate, Tween-20, BME and PVDF membranes were obtained from BIO-RAD (Hercules, CA).

5.3.2 Cell Culture, siRNA-mediated Gene Knockdown and CH-223191

Cells were cultured in DMEM/FBS (10%) and 1% penicillin/streptomycin at 37°C with 5% CO_2. For TCDD (10nM, 24 hours) and ITE (10μM, 72 hours) stimulation, 250,000 MCF7 (ER-positive), T47D (ER-positive), MDA-MB-231 (ER-negative), and MDA-MB-157 (ER-negative) cells were plated on 35mm tissue culture plates in DMEM/FBS (10%) and grown to ~90% confluency. Cells treated with ITE had the media and treatments replenished every 12 hours over the course of three days to compensate for the metabolism of ITE (Cheng, et al., 2015; Henry, Bemis, Henry, Kende, & Gasiewicz, 2006). Transient transfection with siRNA was performed as described in our previous publications (Tomblin & Salisbury, 2014; Tomblin, et al., 2016). In brief, cell suspensions (200,000 cells/mL) were mixed with 100nM of the indicated siRNA with 3μL of Lipofectamine RNAiMAX in DMEM/FBS (10%) for ~20 minutes. Cells were then immediately plated on 35mm tissue culture plates in DMEM/FBS (10%) and cultured for 48 hours before the indicated treatments. Previous reports have shown that CH-223191 is an AHR antagonist that selectively antagonizes AHR ligands with structures similar to TCDD (Kim, et al., 2006; Zhao, Degroot, Hayashi, He, & Denison, 2010). Cells were treated with the indicated concentrations of TCDD or ITE in the absence of presence of 10μM CH-223191 for the indicated periods of time followed by western blot analysis of GAPDH (loading control), JAG1, AHR, Cleaved-NICD, and HES1.

5.3.3 TCDD RNA-sequencing

RNA-seq was conducted as described in our previous report (Salisbury, et al., 2014). Briefly, RNA-seq analysis was based on four biological replicates in each experimental group.

Total RNA purification kits (Qiagen, Valencia, CA) were used to extract total RNA. RNA sample quality was assessed using Bioanalyzer RNA Nano chips (Agilent); all RNA samples had an RIN greater than or equal to 8. RNA-seq libraries were prepared from 1μg of total RNA using a TruSeq RNA Prep Kit (Illumina Inc., San Diego, CA). RNA-seq was performed using an Illumina HiSeq1500 in a 2 x 100 base paired end design yielding a minimum of 50 million reads per sample. Differentially expressed genes were identified at a FDR of 5% as detailed in our prior report (Salisbury, et al., 2014). Raw reads and processed data (un-normalized and normalized read counts by gene) were deposited in the Gene Expression Omnibus (GEO) at the National Center for Biotechnology Information and are accessible via accession number GSE98515. For pathway analysis, TRGs are expressed as a ratio of TCDD/DMSO were loaded into IPA software (Ingenuity Systems, Redwood City, CA). Of the 660 TRGs identified by RNA-seq, 536 were mapped to known functions and pathways by Ingenuity Pathway Analysis (IPA, Qiagen Bioinformatics). The IPA Core Analysis tool was used to identify significant associations between TRGs and curated pathways. To account for testing the TRGs against multiple pathways, we configured IPA Core Analysis to report Benjamini-Hochberg corrected p-values.

5.3.4. RT-qPCR

Total RNA was extracted using RNA purification columns (Qiagen). Reverse transcription was performed on 300ng of RNA using cDNA synthesis kits (Applied Biosystems (Foster City, CA)) in accordance with the suppliers' instructions. Quantitative PCR (qPCR) was conducted with the StepOnePlus (Applied Biosystems) using SYBR green master mix (Applied Biosystems) in accordance with the suppliers' protocols. Samples were run in triplicate and the average was normalized to GAPDH loading control. Relative changes in gene expression was

quantitated using the $2^{-\Delta\Delta CT}$ formula. We have published the GAPDH primer sequences (Salisbury, et al., 2014). JAG1 primers used were: forward 5′-GAGCCCGGCCTCCTTTTATT, reverse 5′-GCGTCATTGTGTTACCTGCG.

5.3.5. Western Blotting

Cells were rinsed with PBS and total cellular extract was isolated in SDS sample buffer (40% glycerol, 8% SDS, 5% BME, 0.04% bromophenol blue in tris-hydrochloride (Tris-HCl), pH 6.8). Proteins were heat denatured and then separated by SDS/PAGE followed by transfer to a PVDF membrane, then blocked with milk (5%) in PBS with 0.05% Tween-20 (PBST). Blots were incubated overnight at 4°C while rocking in primary antibody followed by an incubation period of 90 minutes in secondary antibody at room temperature. Blots were then rinsed five times (5 minutes per rinse) with PBST. Protein targets were detected via chemiluminescence (Millipore, Billerica, MA). JAG1 (1C4), AHR (D5S6H), Cleaved-NICD (D3B8), and HES1 (D6P2U) antibodies were purchased from Cell Signaling Technology (Danvers, MA). GAPDH antibody was purchased from Millipore (Cat #MAB374). Primary antibodies were diluted 1:1000 in PBST. Secondary antibodies were diluted 1:10000 in PBST. ChemiDoc MP Imaging System was used to quantify band density and acquire western blot images.

5.3.6. Scratch Migration Assay

Cells were plated on 12-well tissue culture plates at a concentration of 50,000 cells per well and grown to ~90% confluency prior to treatment. After cells were treated with vehicle or 10μM ITE in DMEM/FBS (1%) every 12 hours for five days, a scratch was made in each well with a pipette tip. Media was then aspirated to removed floating cells, then vehicle or 10μM ITE was reapplied in DMEM/FBS (1%). The scratches were photographed at 0 hours and at 24 hours post scratch using the Lycia Microscope. The exposed surface area of the plates was measured

using ImageJ analysis software. For the indicated siRNA experiments, cell migration was assayed ~48 hours after transfection.

5.3.7. Boyden Chamber Invasion Assay

Cells were plated on 35mm tissue culture plates in DMEM/FBS (10%) for 24 hours prior to treatment. After cells were treated with vehicle or 10μM ITE in DMEM/FBS (10%) every 12 hours for the indicated number of days, they were detached from tissue culture plates (using trypsin) and counted. Cells were immediately transferred to cell invasion chambers in DMEM at a density of 100,000 cells per 500μL. Chambers filled with cells were then incubated in 24-well tissue culture plates containing DMEM/FBS (10%) as the chemoattractant for 36 hours. Each experiment included a negative control in which chambers filled with cells were incubated with DMEM lacking FBS. Following the incubation period in the presence of vehicle (DMSO) or 10μM ITE, a cotton swab was used to remove non-adherent cells that were not invasive. Invasive cells were stained with crystal violet for 10 minutes at room temperature and then rinsed gently with deionized water. Stained cells were then incubated in DMSO for 10 minutes with orbital shaking to extract crystal violet. Cell lysates were measured at 560nm for invasive activity. For the indicated siRNA experiments, the cell invasion was assayed 48 hours after transfection. Boyden cell invasion chambers were purchased from ThermoFisher Scientific.

5.3.8. Statistics

The Tukey's Post-Hoc test was used to determine statistically significant differences among groups following an ANOVA. One-tailed, unpaired t tests with confidence intervals of 95% were used to determine statistically significant differences between two groups.

5.4. Results

5.4.1. TCDD RNA-seq

We have recently shown that treating MCF7 cells with 10nM TCDD for 6 hours changed the expression of 137 genes (Tomblin, et al., 2016). Here, we have analyzed gene expression changes in MCF7 cells treated with 100nM TCDD for 6 hours compared with vehicle controls. Even though the 100nM TCDD concentration may not be consistent with human exposure, the purpose behind the analysis of 100nM TCDD concentrations was to identify robust AHR-regulated target genes in order to elucidate novel AHR-dependent transcriptional mechanisms. This new differential expression-sequencing analysis identified 660 genes that were regulated by 100nM TCDD, including a high representation of genes regulated by 10nM TCDD (123) and genes uniquely regulated by 100nM TCDD (537) (Figure 16). We performed gene expression analyses in IPA to identify biological functions that were most significant to the published TRG and new TRG gene sets. Pathway analysis of the published TRGs (123 genes) revealed enrichment in functions regulated by TCDD, such as cell cycle (Puga, et al., 2000), cell death and survival (Bekki, et al., 2015), lipid metabolism (Bui, Solaimani, Wu, & Hankinson, 2012; Yang, Solaimani, Dong, Hammock, & Hankinson, 2013) and drug metabolism (Nebert & Dalton, 2006) (Table 5), while the new TRGs (537 genes) were enriched in pathways associated with other aspects of breast cancer (Table 6). These included cell morphology (Diry, et al., 2006), cell assembly and organization, cell-to-cell signaling, cell development and cellular movement (Hall, et al., 2010; Zhang, et al., 2012), signifying a potential broader role for TCDD in breast cancer.

TRGs can be roughly divided into two groups. One group is directly regulated by TCDD via the AHR binding to DREs, and the other group is indirectly regulated by TCDD because the

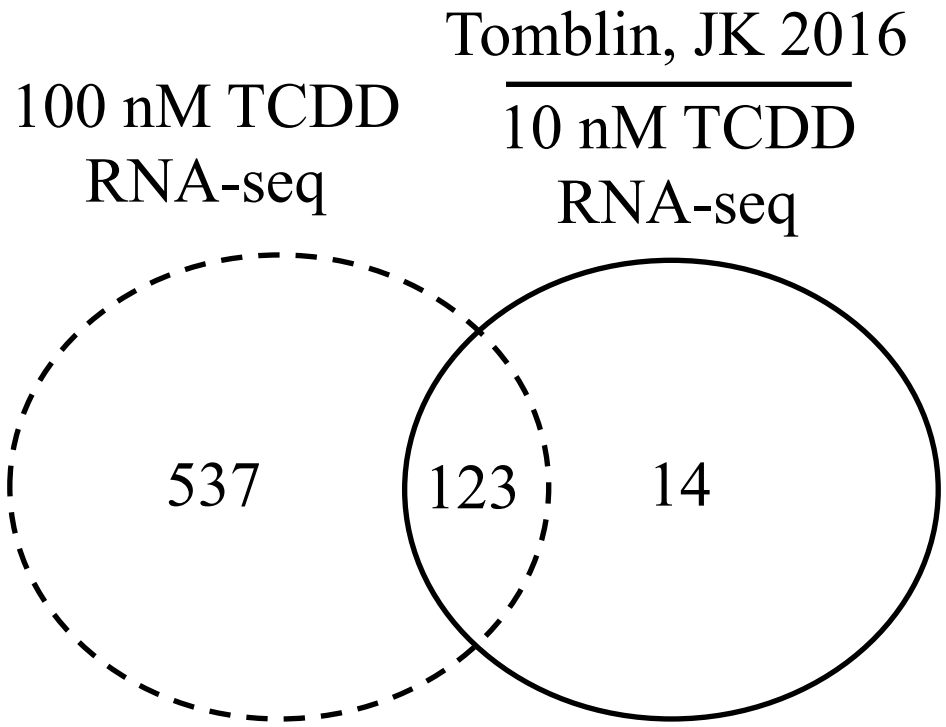


Figure 16. Comparison of Published 10nM TRGs with the More Extensive List of 100nM TRGs

Analysis of the 100nM TRGs showed enrichment for published TRGs (123) (Tomblin, et al., 2016) and new TRGs (537). Genes from the 10nM TCDD-treated MCF7 cells are highly represented in the 100nM TCDD-treated MCF7 cells gene set.

IPA Biofunctions Associated with 123 Published TRGs from 10nM and 100nM TCDD-treated MCF7 Cells		
Category	***B-H p-value**	**# TRGs**
Cell Cycle	1.34E-03-2.79E-02	18
Cell Death and Survival	3.03E-03-2.79E-02	32
Lipid Metabolism	5.18E-03-2.79E-02	13
Small Molecule Biochemistry	5.18E-03-2.79E-02	26
Cell Morphology	5.31E-03-2.79E-02	30
Cell Function and Maintenance	5.31E-03-2.79E-02	23
Gene Expression	6.8E-03-2.79E-02	12
Carbohydrate Metabolism	7.54E-03-2.79E-02	12
Drug Metabolism	7.54E-03-2.79E-02	9
Energy Production	7.54E-03-2E-02	7
*p-values are calculated by Fishers exact test and corrected for multiple testing by the Benjamini-Hochberger p-values (B-H) method (B-H p-value). Column 2 shows the range of B-H corrected p-values for the biofunction in a given category.		

Table 5. IPA Biofunctions Associated with 123 TRGs from 10nM and 100nM TCDD-treated MCF7 Cells

Biofunctional analysis was conducted on the 123 overlapped genes to identify if there are any associated biofunctions related to cancer biology. Column 1 identifies the biofunctional category that IPA linked to the 123 TRGs. Column 2 shows the range of B-H corrected p-values for the biofunction in a given category and the strength of biofunctional association is ordered from top to bottom. Cancer-related biofunctions found in this table include cell morphology, cell death and survival, cell-to-cell signaling, cell movement, cell cycle, cell development, cell growth and proliferation, and cell assembly and organization. Column 3 identifies the number of genes within the dataset to be associated within the given biofunctional category.

IPA Biofunctions Associated with 537 Uniquely Regulated Genes from 100nM TCDD-treated MCF7 Cells		
Category	***B-H p-value**	**# TRGs**
Cell Morphology	1.14E-06-1.31E-02	111
Cell Assembly and Organization	1.14E-06-9.46E-03	81
Cell Function and Maintenance	1.14E-06-9.46E-03	114
Cell-To-Cell Signaling	4.87E-06-1.31E-02	66
Cell Development	1.1E-05-1.44E-02	128
Cell Growth and Proliferation	1.1E-05-1.31E-02	120
Amino Acid Metabolism	1.45E-05-3E-03	18
Post-Translational Modification	1.45E-05-1.28E-03	41
Small Molecule Biochemistry	1.45E-05-1.44E-02	46
Cellular Movement	2.63E-04-1.38E-02	83

*p-values are calculated by Fishers exact test and corrected for multiple testing by the Benjamini-Hochberger p-values (B-H) method (B-H p-value). Column 2 shows the range of B-H corrected p-values for the biofunction in a given category.

Table 6. IPA Biofunctions Associated with 537 Uniquely Regulated Genes from 100nM TCDD-treated MCF7 Cells

Biofunctional analysis was conducted on the 537 regulated genes from the 100nM TCDD-treated MCF7 cells in order to identify if there are any associated biofunctions related to cancer biology. Column 1 identifies the biofunctional category that IPA linked to the 123 TRGs. Column 2 shows the range of B-H corrected p-values for the biofunction in a given category and the strength of biofunctional association is ordered from top to bottom. Column 3 identifies the number of genes within the dataset to be associated within the given biofunctional category. Cancer-related biofunctions found in this table include cell morphology, cell assembly and organization, cell-to-cell signaling, cell development, cell growth and proliferation, and cellular movement. Because this biofunctional analysis contained categories that were more relevant to cancer biology compared to the 123 overlapped gene set (Table 5), bioinformatic and genomic analysis was conducted with the 100nM TCDD gene set.

gene does not possess DREs; however, they are part of the TCDD pathway. Previously, 2,594 TCDD-stimulated AHR binding sites were identified in MCF7 cells by ChIP-seq (Lo & Matthews, 2012). We overlapped these published 2,594 TCDD-AHR binding sites (Lo & Matthews, 2012) with our complete TRGs set and identified that 189 genes were common between the two groups (Figure 17). The 189 shared genes represent probable direct TCDD-stimulated AHR gene targets (Table 7), while the remaining 471 TRGs are regulated indirectly by TCDD-stimulated AHR activation. IPA biological function analysis of the primary 189 TRGs showed enrichment in functions downstream of TCDD including gene expression, cell cycle (Barhoover, Hall, Greenlee, & Thomas, 2010; Puga, et al., 2000; Marlowe, Knudsen, Schwemberger, & Puga, 2004), cell death and survival (Bekki, et al., 2015), cell morphology (Diry, et al., 2006), cell-to-cell signaling, cellular movement (Hall, et al., 2010; Zhang, et al., 2012), cellular development and cell assembly and organization, all of which provide insight into how TCDD may act in breast cancer (Table 8).

We filtered this list to binding sites that appeared less than 5000 bases upstream of an annotated TSS in order to identify direct AHR target genes. This analysis identified 224 genes that had TCDD-stimulated AHR binding sites identified by ChIP-Seq (Lo & Matthews, 2012) less than 5 kilobases (Kb) upstream of their TSS. We compared this list of 224 genes with our complete list of TRGs (660) and identified that 41 were common (Figure 18). The 41 shared genes represent gene targets where the TCDD-AHR binding site is within 5Kb of the TSS (Table 9), while the remaining 619 TRGs are regulated either indirectly by TCDD-AHR or the TCDD-AHR binding site is greater than 5Kb of the TSS.

Next, an IPA upstream regulator analysis was used to determine if genes within the 100nM TRG set (660 genes) were regulated through a common upstream regulator (Table 10).

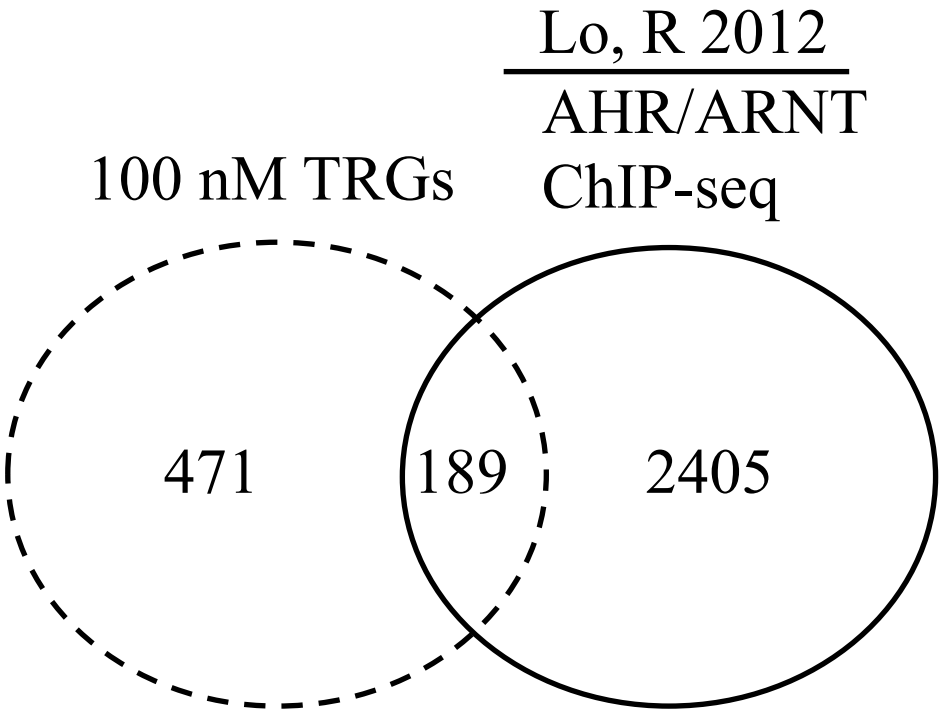


Figure 17. Comparison of Published 100nM TRGs with TCDD-stimulated AHR Binding Sites Identified by ChIP-seq in MCF7 Cells

To better understand AHR-ARNT signaling in breast cancer cells, Lo et al. used chromatin immunoprecipitation linked to high-throughput sequencing to identify AHR and ARNT binding sites across the genome in TCDD-treated MCF-7 cells (Lo & Matthews, 2012). They identified 2594 AHR-bound regions, which were overlapped with the 100nM TRGs analysis to determine any potential direct AHR target genes. This overlap identified 189 genes that are directly bound by AHR when stimulated by TCDD which are displayed in Table 7.

The 189 TRGs that Overlap with Reported TCDD-stimulated AHR-ChIP-seq from Lo & Matthews (2012)											
Gene Name	***Fold Change**	**Gene Name**	***Fold Change**	**Gene Name**	***Fold Change**	**Gene Name**	***Fold Change**	**Gene Name**	***Fold Change**	**Gene Name**	***Fold Change**
CYP1A1	713.9	TMTC2	2.5	ELF4	2.0	TUFT1	1.6	CCDC88C	1.4	CD47	1.3
CYP1B1	12.5	TNFRSF11B	2.4	FAM198B	1.9	RRS1	1.6	LPP	1.4	FREM2	1.3
DRD1	8.0	NEDD9	2.4	FAM110B	1.9	PKP1	1.6	SIPA1L2	1.4	NPEPPS	1.3
PITPNM2	6.7	C1orf116	2.4	PRDM16	1.8	LRIG3	1.6	ABCC5	1.4	GMNN	1.3
STRA6	6.0	ITPR1	2.3	PHEX	1.8	INPP4B	1.6	SIPA1L3	1.4	C4orf32	1.3
TRPM6	5.8	TSKU	2.3	ATP1B1	1.8	NEK6	1.5	FARP1	1.4	SSBP2	1.3
LMCD1	5.5	ZIC2	2.3	SLC16A14	1.8	FRMD4A	1.5	TRAFD1	1.4	RCC2	1.3
SLC7A5	5.0	DNMBP	2.2	DLL1	1.8	DDIT4	1.5	PDZRN3	1.4	TRIO	1.3
ALDH1A3	4.2	STC2	2.2	FOSL2	1.8	PSPC1	1.5	RAP1GAP	1.4	PARP4	1.3
ABCG2	4.2	AGPAT9	2.2	GAD1	1.8	GLI3	1.5	MSX2	1.4	SPTBN1	1.3
HAR1A	3.8	PYGL	2.2	HES1	1.8	IL1RAPL2	1.5	NR3C1	1.4	MED13L	1.3
NCCRP1	3.8	ATP9A	2.2	NRP1	1.8	ADAP1	1.5	USP3	1.4	SLC4A7	1.3
RUNX2	3.8	FAM84A	2.2	SAMD12	1.8	NADK	1.5	CDC25B	1.4	MYO6	1.3
HAR1B	3.7	CABLES1	2.2	C9orf3	1.8	DSP	1.5	MEIS1	1.4	TRPS1	1.2
BMF	3.6	SALL4	2.2	AHRR	1.7	SOS1	1.5	MTMR2	1.4	HBP1	0.8
HAR1A	3.6	LEKR1	2.2	SLC2A11	1.7	REL	1.5	IER5	1.4	HDAC5	0.8
ST3GAL1	3.4	NFE2L2	2.2	SLC27A2	1.7	GDF15	1.5	SPAG17	1.4	MYB	0.8
GLDN	3.3	HS6ST3	2.1	RUNX1	1.7	EGFR	1.5	RIOK1	1.4	GPRC5A	0.8
RND1	3.2	GPR115	2.1	KLF7	1.7	IGF1R	1.5	PIK3C2B	1.4	KITLG	0.8
LEF1	3.1	VIPR1	2.1	ZFHX3	1.7	NHS	1.5	ROCK2	1.3	TESK2	0.8
FAM105A	3.0	LRP5	2.1	SCARA5	1.7	C15orf39	1.5	TSHZ2	1.3	KIAA1467	0.7
TIPARP	3.0	ADAM12	2.1	VDR	1.7	SPRED2	1.5	LPHN2	1.3	FAM5B	0.7
C9orf169	3.0	CLSTN2	2.1	SHISA2	1.7	GPR157	1.5	CPEB4	1.3	AGR3	0.7
MAPRE2	2.9	NBPF1	2.0	TMEM120B	1.6	HOXC11	1.5	GNL3	1.3	CSRNP1	0.7
SYT12	2.8	KIAA1549	2.0	ACOXL	1.6	PTPRJ	1.5	QSOX1	1.3	LYN	0.7
TTC39B	2.8	PPARG	2.0	TNS3	1.6	DOCK5	1.5	PSD3	1.3	NPY1R	0.7
PLEKHF1	2.7	WNK2	2.0	PRPS1	1.6	CARD10	1.5	ARHGEF10L	1.3	SLC30A1	0.7
MCOLN2	2.6	WSCD1	2.0	ZBTB7C	1.6	SPIRE2	1.5	PAK2	1.3	RBM24	0.7
HECW2	2.6	RARRES1	2.0	TDH	1.6	BMPR1B	1.4	SYDE2	1.3	GSC	0.6
EDC3	2.6	DUSP10	2.0	TIMP3	1.6	JARID2	1.4	EHD4	1.3	MAF	0.6
HEY1	2.5	SLC16A7	2.0	SNX25	1.6	CUX1	1.4	BOD1	1.3	TP73	0.5
TMEM45B	2.5			TPCN1	1.6			DPP9	1.3		

*RNA-fold change expression values from 100 nM TCDD-treated MCF7 cells compared with vehicle expressed as a ratio/vehicle

Table 7. The 189 TRGs that Overlap with Reported TCDD-stimulated AHR-ChIP-seq from Lo & Matthews (2012)

IPA Biofunctions Associated with 189 Primary TRGs from the 100nM TCDD-treated MCF7 Cells Overlapped with ChIP-seq Analysis from Lo & Matthews (2012)		
Category	***B-H p-value**	**# TRGs**
Cell Morphology	3.83E-06-1.37E-02	60
Cell Death and Survival	2.58E-05-8.62E-03	63
Cell-To-Cell Signaling	2.66E-05-1.14E-02	33
Cell Movement	3.15E-05-1.36E-02	53
Cell Cycle	3.33E-05-1.41E-02	46
Cell Development	3.84E-05-1.29E-02	69
Gene Expression	8.38E-05-7.81E-03	50
Cell Function and Maintenance	1.2E-04-1.37E-02	63
Cell Growth and Proliferation	3.41E-04-1.17E-02	70
Cell Assembly and Organization	1.48E-03-1.37E-02	39
*p-values are calculated by Fishers exact test and correct for multiple testing by the Benjamini-Hochberger p-values (B-H) method (B-H p-value).		

Table 8. IPA Biofunctions Associated with 189 Primary TRGs from the 100nM TCDD-treated MCF7 Cells Overlapped with ChIP-seq Analysis from Lo & Matthews (2012)
Biofunctional analysis was conducted on the 189 primary TRGs in order to identify if there are any associated biofunctions related to cancer biology. Column 1 identifies the biofunctional category that IPA linked to the 189 TRGs. Column 2 shows the range of B-H corrected p-values for the biofunction in a given category and the strength of biofunctional association is ordered from top to bottom. Cancer-related biofunctions found in this table include cell morphology, cell death and survival, cell-to-cell signaling, cell movement, cell cycle, cell development, cell growth and proliferation, and cell assembly and organization. Column 3 identifies the number of genes within the dataset to be associated within the given biofunctional category.

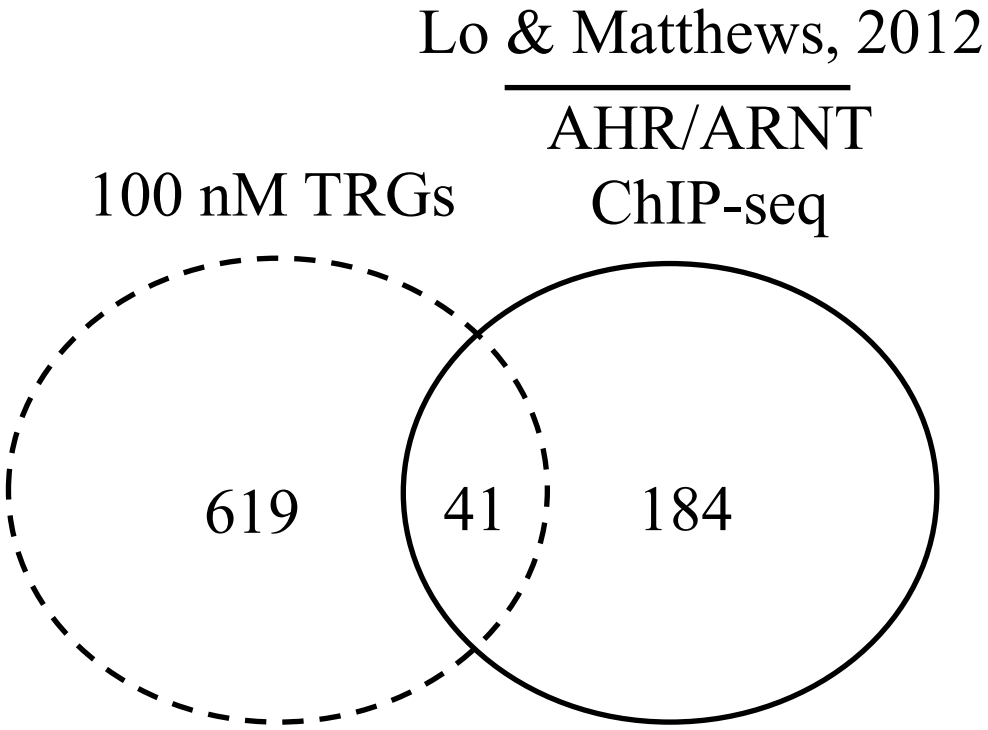


Figure 18. Comparison of Published 100nM TRGs with TCDD-stimulated AHR Binding Sites Identified by ChIP-seq Less than 5Kb Upstream of their TSS
The identified 2594 AHR-bound regions via ChIP-seq analysis (Lo & Matthews, 2012) were filtered down to genes that contained AHR-binding sites within 5Kb of the TSS, in order to determine bonafide direct AHR target genes. This analysis identified 224 genes that met the criteria and were then overlapped with the 100nM TCDD RNA-seq to determine what genes may be directly targeted by AHR within our gene set. This overlap identified 41 genes that are directly bound by AHR and are within 5Kb of the transcription start site which are displayed in Table 9.

The 41 Genes Within the 100nM TCDD RNA-seq Data Set with an AHR Binding Site Within 5Kb of the TSS in MCF7 Cells			
Gene Name	***Fold Change**	**Gene Name**	***Fold Change**
CYP1A1	713.9	RUNX1	1.7
CYP1B1	12.5	VDR	1.7
LMCD1	5.5	PRPS1	1.6
ABCG2	4.2	LSMEM1	1.6
NCCRP1	3.8	TUFT1	1.6
C9orf169	3.0	DDIT4	1.5
SYT12	2.8	ADAP1	1.5
RNF224	2.8	NADK	1.5
PLEKHF1	2.7	SOS1	1.5
TMTC2	2.5	REL	1.5
AC018816.3	2.4	IGF1R	1.5
ITPR1	2.3	CARD10	1.5
ZIC2	2.3	USP3	1.4
SALL4	2.2	CPEB4	1.3
PPARG	2.0	DPP9	1.3
FAM198B	1.9	FREM2	1.3
SLC34A3	1.8	SSBP2	1.3
GAD1	1.8	SYBU	0.7
HES1	1.8	RBM24	0.7
NRP1	1.8	TP73	0.5
SLC2A11	1.7		
*RNA-fold change expression values from TCDD-treated MCF7 cells compared with vehicle expressed and a ratio/vehicle			

Table 9. The 41 Genes Within the 100nM TCDD RNA-seq Data Set with an AHR Binding Site Within 5Kb of the TSS in MCF7 Cells

IPA Upstream Regulators Associated with 100nM TRGs (660)				
Upstream Regulator	**Predicted Activation State**	***p-value of Overlap**	**Target Genes in Dataset**	**Number of Genes in the Network**
Beta-estradiol		1.8E-12	87	203
PGR		5.4E-10	26	199
SOX2		2E-09	27	131
Progesterone	Activated	1.6E-08	37	184
TP63		3.2E-08	28	182
TNF		7.4E-08	73	219
Valproic Acid		3.5E-07	29	170
Estrogen Receptor		3.7E-07	20	179
Fulvestrant		4.4E-07	21	185
NOTCH1	Activated	5.1E-07	20	163
AHR	Activated	6.1E-06	22	162
TCDD	Activated	8.3E-05	21	155
*p-value of overlap are calculated by Fisher exact test				

Table 10. IPA Upstream Regulators Associated with 100nM TRGs (660)
An IPA upstream regulator analysis was conducted to identify transcription factors and other upstream molecules that may be responsible for gene expression changes observed. Column 1 indicates the transcription factors and or small molecules that IPA predicted to be associated with the 100nM TCDD TRGs. Column 2 uses the activation z-score algorithm to predict IPA upstream regulator activity, which is determined by comparing reported gene responses to a given upstream regulator to the observed expression changes in 100nM TRGs. Column 3 shows the strength of overlap and is ordered from top to bottom, based on the calculated p-value by the Fisher exact test. Column 4 shows the number of TRGs in the RNA-seq 100nM TCDD dataset that are within the gene network under a specific IPA-upstream regulator. Column 5 indicates the total number of genes in the network under a specific IPA upstream regulator pathway.

This analysis revealed that TRGs were significantly enriched under the following upstream regulators: beta-estradiol (endogenous hormone), PR (ligand-activated transcription factor), SRY box 2 (SOX2) (transcription factor), progesterone (endogenous hormone), tumor protein 63 (TP63) (transcription factor), tumor necrosis factor (TNF) (cytokine), valproic acid (chemical drug), ER (ligand-activated transcription factor), fulvestrant (chemical drug), Notch1 (transcription factor), AHR (ligand-activated transcription factor) and TCDD (chemical toxicant) (Table 10). Of the 37 TRGs in the progesterone pathway, 21 exhibited patterns of expression consistent with activation of progesterone signaling activity (Table 10). For Notch1, the pattern of expression of 13 of the 20 TRGs was consistent with activation of the Notch1 pathway (Table 11).

In ER-expressing breast cancer cells (e.g. MCF7 and T47D), TCDD increases: 1) posttranscriptional downregulation of the ER (Wormke, et al., 2003), 2) the binding of the AHR to inhibitory dioxin response elements (iDREs) that perturb the binding of ER and associated transcriptional activators to ER target genes (Krishnan, Porter, Santostefano, Wang, & Safe, 1995), 3) the co-recruitment of AHR and ER complexes to the promoters of AHR and ER target genes (Beischlag & Perdew, 2005; Matthews, Wihlen, Thomsen, & Gustafsson, 2005) and 4) the recruitment of receptor interacting protein 140 (RIP140) (negative transcriptional regulator) to certain ER target genes that are suppressed by TCDD-stimulated AHR signaling (Madak-Erdogan & Katzenellenbogen, 2012). Based on the findings of these prior reports, identifying that 87 of 203 beta-estradiol target genes are TRGs is not surprising. Finally, TRGs were significantly enriched within the AHR (20 of 162 AHR network genes) and TCDD pathways (21 of 155 TCDD network genes), which is consistent with cells being treated with TCDD. The IPA-predicted AHR activation and TCDD activity was based in part on the induction of canonical

TCDD-stimulated AHR target genes CYP1A1 (~ 714-fold) (Spink, et al., 1998), CYP1B1 (~12.5-fold) (Spink, et al., 1998) and ALDH3A1 (~ 4.2-fold) (Vasiliou, Reuter, Williams, Puga, & Nebert, 1999) in TCDD-treated cells compared with controls (Table 7).

The 20 TRGs that are grouped under the IPA-defined Notch1 network are shown in Table 11. Overlap analysis with predicted direct TCDD-AHR regulated genes (Table 7) shows that half of the genes within the Notch1 network were predicted direct TCDD-AHR gene targets and the other half were indirect TCDD-AHR gene targets that did not bind the AHR (Table 11). The predicted direct TCDD-AHR gene targets that are also Notch1 network genes include RUNX2 (Hilton, et al., 2008), v-rel avian reticuloendotheliosis viral oncogene homolog (c-Rel) (Cheng, et al., 2001), PPARγ (Sciaudone, Gazzerro, Priest, Delany, & Canalis, 2003), MYC proto-oncogene (myc) (Weng, et al., 2006), lymphoid enhancing factor 1 (LEF1) (Spaulding, et al., 2007), IGF-1R (Eliasz, et al., 2010), HEY1 (Borggrefe & Liefke, 2012), HES1 (Borggrefe & Liefke, 2012), EGFR (Baumgart, et al., 2010), and DLL1 (Jaleco, et al., 2001). Overlap analysis with TCDD-stimulated AHR regulated genes that have TCDD-AHR binding sites within 5Kb of the TSS (Table 9) shows that four TRGs that meet these criteria are within the Notch1 network. Those genes include c-Rel (Cheng, et al., 2001), PPARγ (Sciaudone, Gazzerro, Priest, Delany, & Canalis, 2003), IGF-1R (Eliasz, et al., 2010), and HES1 (Borggrefe & Liefke, 2012) (Table 9). The group of 10 Notch1 network genes that are regulated by TCDD-AHR through an indirect mechanism included JAG1 (Table 9). JAG1 is the predominant Notch receptor ligand in TNBC that is also implicated in breast cancer progression (Reedijk, et al., 2005; Dickson, et al., 2007; Cohen, et al., 2010). Because JAG1 is clinically linked to breast cancer progression (Dickson, et al., 2007; Reedijk, et al., 2005) (Figure 10), we investigated the mechanism by which TCDD reduces the expression of JAG1.

TRGs (100 nM) that are IPA-determined Notch1 Regulated Genes				
Genes in Dataset	**Predicted State of Activation**	**Expression Log Ratio of TRGs**	**Predicted TRG**	**TRG Within 5Kb of TSS**
RUNX2	Activated	1.914	Yes	No
REL	Activated	0.567	Yes	Yes
PPARG	Activated	1.021	Yes	Yes
NCF1	Activated	-0.518	No	No
MYC	Activated	0.881	Yes	No
LEF1	Activated	1.645	Yes	No
IGFBP3	Activated	-0.628	No	No
IGF1R	Activated	0.56	Yes	Yes
HEY1	Activated	1.336	Yes	No
HES1	Activated	0.852	Yes	Yes
EGFR	Activated	0.561	Yes	No
DKK1	Activated	-0.729	No	No
CHST1	Activated	-1.005	No	No
MYCL	Inhibited	-0.568	No	No
CAD	Affected	0.586	No	No
DLL1	Affected	0.872	Yes	No
ITGA6	Affected	-0.399	No	No
JAG1	Affected	-0.336	No	No
LFNG	Affected	-0.678	No	No
WNT5A	Affected	1.998	No	No

Table 11. TRGs (100nM) that are IPA-determined Notch1 Regulated Genes
According to the IPA upstream regulator analysis, Notch1 was predicted to be activated based on the gene expression changes present within the 100nM TRGs (660) and shows 20 genes that are downstream of the Notch signaling pathway to be affected in the presence of TCDD. Column 1 shows the name of the 100nM TRG. Column 2 is the predicted activation state of Notch signaling based on the direction of gene expression change. Column 3 shows the fold change of TRGs. Column 4 shows if the gene is a direct AHR target gene based from overlapping 100nM TCDD RNA-seq with TCDD-AHR ChIP-seq (Table 5). Column 5 identifies primary TRGs that bind to TCDD-AHR binding sites within 5Kb of transcription start site (Table 7).

5.4.2. TCDD Reduces JAG1 mRNA in MCF7 Cells

In an effort to confirm the RNA-seq analysis, we treated MCF7 cells with DMSO or the indicated concentrations of TCDD for 6 hours. The relative mRNA levels of JAG1 normalized to GAPDH loading control were determined by RT-qPCR. We verified that JAG1 expression was reduced by TCDD and found that 10nM and 100nM TCDD treatment significantly reduced JAG1 mRNA (by ~40%) in MCF7 cells after 6 hours of treatment (Figure 19).

5.4.3. TCDD Reduces JAG1 Protein but Activates Notch Signaling in MCF7 Cells

After establishing that TCDD significantly reduced JAG1 mRNA levels after 6 hours in MCF7 cells, we sought to determine if TCDD also reduced the levels of JAG1 protein and whether changes in the levels of JAG1 were correlated with changes in the activity of the Notch signaling pathway. We, therefore, treated MCF7 cells with 10nM TCDD for 24 hours and extracted total cellular protein to analyze by western blot (Figure 20). We probed blots with JAG1, AHR, and HES1 antibodies. The activation of Notch signaling induces the proteolysis of the Notch receptor, and this releases the NICD, which functions as a transcription factor that activates Notch target genes like HES1 (Borggrefe & Liefke, 2012). We, therefore, also probed blots with an antibody that specifically recognizes activated cleaved-NICD as the readout for changes in Notch signaling. The western blot signals for JAG1, AHR, HES1, and cleaved-NICD were normalized to GAPDH. We have shown in prior reports that GAPDH is a suitable loading control for western blots analysis because its levels are not changed in response to AHR ligands (Salisbury, et al., 2014).

The western blot findings showed that after 24 hours TCDD significantly reduced the levels of AHR protein compared with vehicle (Figure 20). The observed reduction in AHR in response to TCDD is consistent with prior reports that have shown that TCDD-activated AHR is

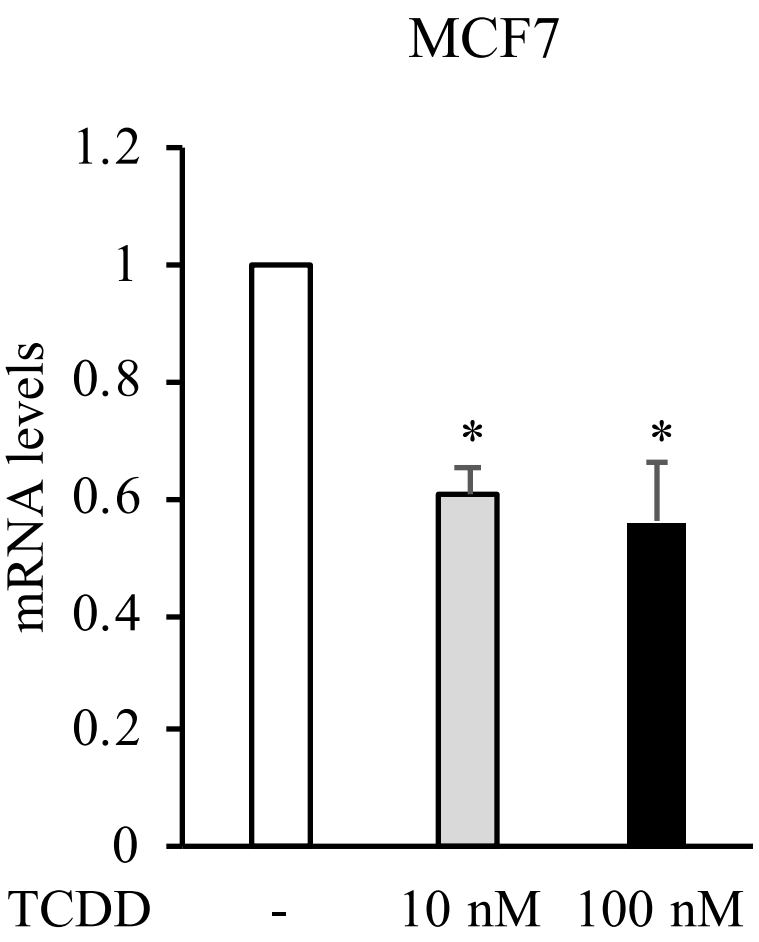


Figure 19. TCDD Decreases JAG1 mRNA Levels in MCF7 Cells
MCF7 cells were treated with vehicle or the indicated concentrations of TCDD for 6 hours. The relative mRNA levels of JAG1 normalized to GAPDH loading control were determined by RT-qPCR (as detailed in the Material and Methods). Results shown are the means ± standard error margin (SEM) of three independent experiments. Significant reductions by TCDD are indicated by *$P < 0.05$.

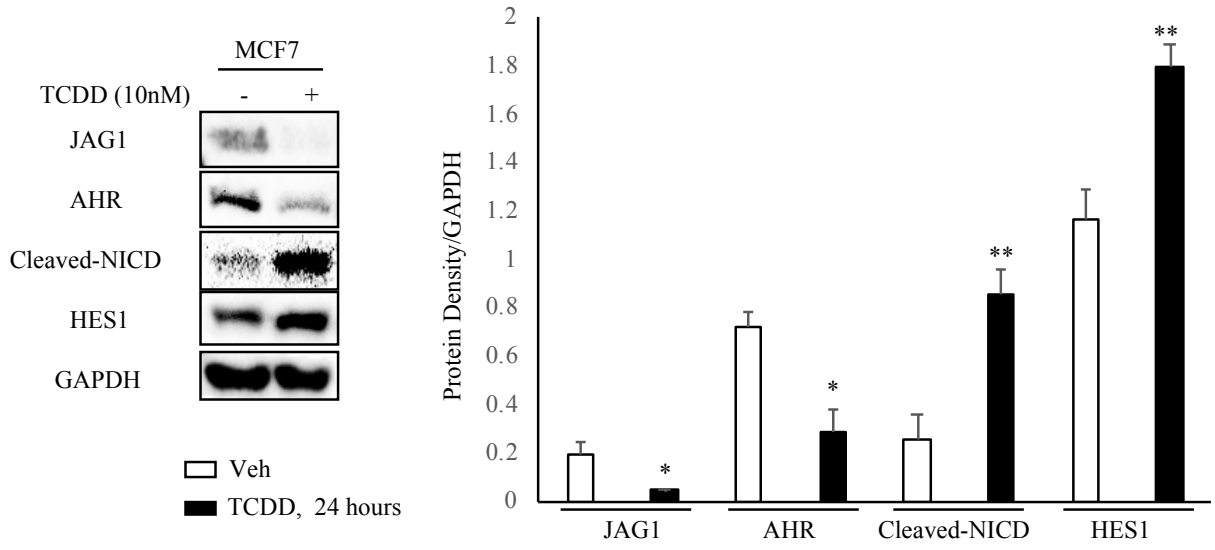


Figure 20. TCDD Decreases JAG1 Protein Levels but Activates the Notch Signaling Pathway in MCF7 Cells

MCF7 cells were treated with vehicle or 10nM TCDD for 24 hours. Total cellular protein was isolated and subjected to western blot analysis. Blots were probed with the indicated antibodies. Levels of JAG1, AHR, Cleaved-NICD, and HES1 were normalized to GAPDH. Results shown are the means ± SEM of three independent experiments. Significant changes in expression by TCDD are indicated by *P < 0.05 or **P<0.01.

ubiquitinated and then degraded by the proteasome and that this regulation is a negative-feedback loop in the TCDD signaling pathway (Ma & Baldwin, 2000). We also observed TCDD-stimulated reductions in the levels of JAG1 protein (Figure 20), which is consistent with our RNA-seq and RT-qPCR results showing that TCDD reduced the expression of JAG1 mRNA. We hypothesized that TCDD-induced reductions in JAG1 would lead to the attenuation of Notch signaling, because JAG1 is a Notch receptor ligand. The findings, however, revealed that TCDD-stimulated a robust increase in the levels of NICD (Figure 20). This result indicates that in ER-expressing MCF7 cells, TCDD is sufficient to activate the Notch pathway, even when the levels of JAG1 protein are reduced in response to TCDD (Figure 20). To further investigate whether TCDD-stimulated increases in the NICD have a functional impact in MCF7 cells, we evaluated the expression of HES1, which is a direct Notch target gene (Borggrefe & Liefke, 2012). In accordance with the observed increases of cleaved-NICD levels in response to TCDD, we identified that TCDD stimulated HES1 expression in MCF7 cells (Figure 20). Collectively, these results indicate that TCDD is sufficient to increase Notch signaling, because it induced cleaved-NICD levels and promoted the expression of the Notch target gene, HES1. TCDD, however, reduced the expression of the Notch receptor ligand JAG1 at the level of mRNA and protein.

5.4.4. TCDD Reduces JAG1 mRNA in MDA-MB-231 Cells

We sought to further investigate the regulation of JAG1 and Notch signaling by TCDD in MDA-MB-231 TNBC cells. The impetus for including MDA-MB-231 cells in our TCDD analysis stems from prior reports showing that the JAG1-Notch pathway is more active in TNBC compared with ER-positive breast cancer. We first evaluated the levels of JAG1 mRNA, and the findings showed that treating MDA-MB-231 cells with 10nM TCDD for 24 hours significantly reduced the levels of JAG1 mRNA compared with cells treated with vehicle (Figure 21).

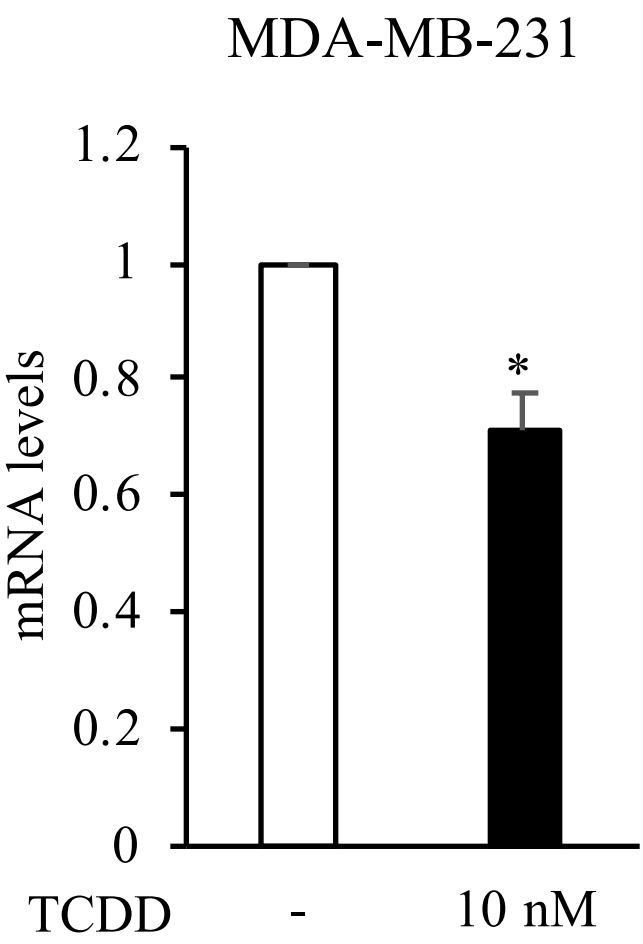


Figure 21. TCDD Decreases JAG1 mRNA Levels in MDA-MB-231 Cells
MDA-MB-231 cells were treated with vehicle or 10nM TCDD for 6 hours. The relative mRNA levels of JAG1 normalized to GAPDH loading control was determined by RT-qPCR (as detailed in the Material and Methods). Results shown are the means ± SEM of three independent experiments. Significant reductions by TCDD are indicated by *P < 0.05.

5.4.5. TCDD Reduces JAG1 Protein and Inhibits Notch Signaling in MDA-MB-231 Cells

Having established that TCDD significantly reduces the expression of JAG1 mRNA, we sought to investigate whether this prototype AHR ligand also reduces the levels of JAG1 protein in MDA-MB-231 cells. The western blot findings showed that 10nM TCDD (24 hours) significantly reduced the levels of JAG1 protein in MDA-MB-231 cells, which is similar to what we had observed in MCF7 cells (Figure 22). Next, we investigated Notch signaling activity by measuring the cleaved-NICD levels by western blot in MDA-MB-231 cells treated with vehicle or 10nM TCDD (24 hours). In accordance with prior reports, the levels of cleaved-NICD were high in control MDA-MB-231 cells (Figure 22). This result indicates that MDA-MB-231 cells exhibit constitutive Notch signaling under basal conditions, which is different than what we observed in MCF7 cells. As shown in Figure 20, control MCF7 cells do not express cleaved-NICD under basal conditions. The western blot findings also revealed that TCDD-stimulated reductions in levels of cleaved-NICD in MDA-MB-231 cells (Figure 22). Collectively, these results indicate that TCDD-activated AHR signaling reduces JAG1 and cleaved-NICD levels in the TNBC MDA-MB-231 cell line.

5.4.6. AHR Mediates TCDD-stimulated Reductions in JAG1 and Cleaved-NICD in MDA-MB-231 Cells

We performed AHR knockdown experiments in MDA-MB-231 cells to determine whether this receptor mediates TCDD-induced downregulation of JAG1. Control cells were transfected with control siRNA that was non-targeting. As anticipated, TCDD significantly reduced JAG1 protein (by ~ 44%) in control cells (Figure 23A). Western blot analysis of cells transfected with AHR-targeting siRNA revealed that AHR was significantly reduced (by ~ 95%)

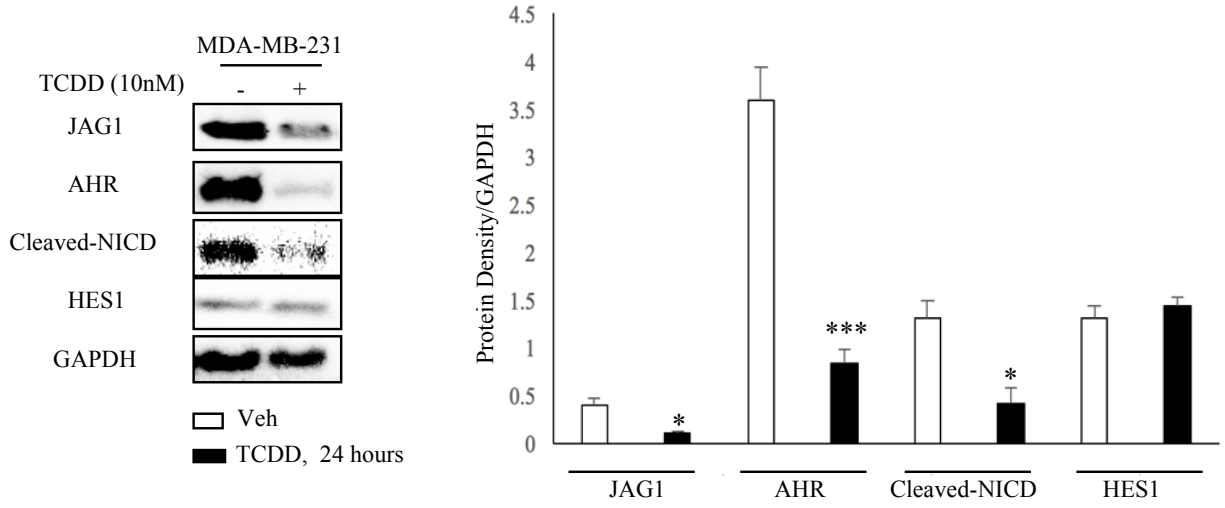


Figure 22. TCDD Decreases JAG1 Protein Levels and Inhibits the Notch Signaling Pathway in MDA-MB-231 Cells

MDA-MB-231 cells were treated with vehicle or 10nM TCDD for 24 hours. Total cellular protein was isolated and subjected to western blot analysis. Blots were probed with the indicated antibodies. Levels of JAG1, AHR, Cleaved-NICD, and HES1 were normalized to GAPDH. Results shown are the means ± SEM of three independent experiments. Significant changes in expression by TCDD are indicated by *P < 0.05 or ***P<0.001.

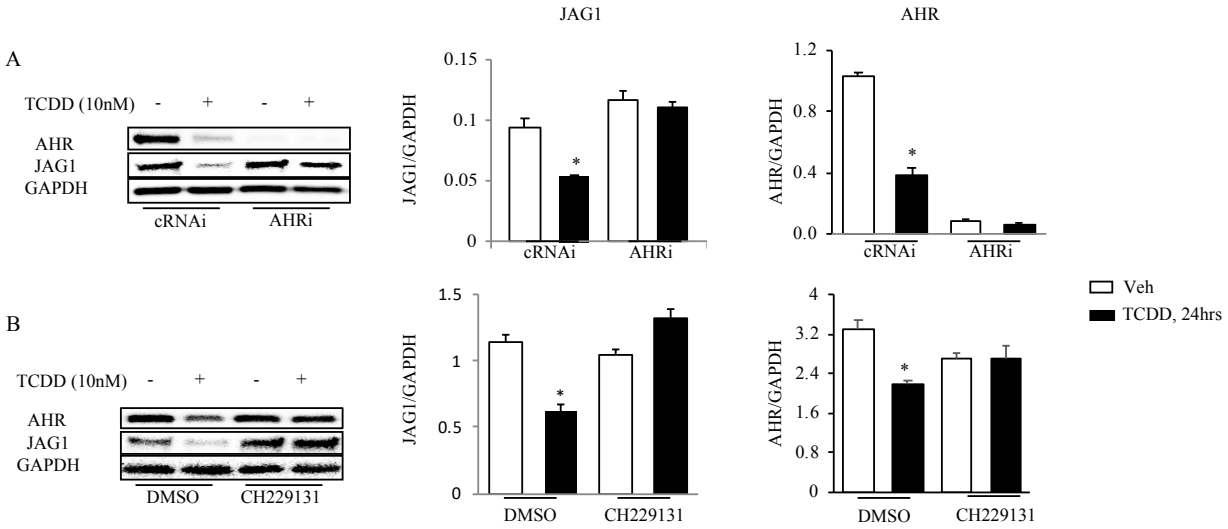


Figure 23. AHR Mediates TCDD-stimulated Reductions of JAG1 in MDA-MB-231 Cells
A) Control cells were transfected with non-targeting short interfering RNA and AHR knockdown cells were transfected with AHR-targeting siRNA. Forty-eight hours after transfection, cells were treated with vehicle or 10nM TCDD for 24 hours. B) Control cells were treated with vehicle or 10nM TCDD. CH-223191 groups were treated with 10μM CH-223191 in the absence or presence of 10nM TCDD for 24 hours. Total cellular protein was isolated and subjected to western blot analysis. Blots were probed with the indicated antibodies. Levels of AHR and JAG1 were normalized to GAPDH. Data shown are the means ± SEM of three independent experiments. Significant reductions by TCDD or AHR-targeting siRNA are indicated by * $P < 0.05$.

compared to control cells, indicating that our approach to knocking down AHR was highly effective (Figure 23A). We observed that cells with AHR knockdown were unresponsive to TCDD-stimulated reductions in JAG1, indicating that the suppressive effect of TCDD on JAG1 expression is dependent on AHR activation (Figure 23A). The observed reduction in AHR protein in response to TCDD in control cells (Figure 23A) is mediated by a posttranscriptional mechanism that increases the ubiquitination of AHR, which in turn causes AHR to be degraded by the proteasome (Wormke, et al., 2003; Pollenz, 2002). The AHR antagonist CH-223191 is reported to selectively antagonize the binding of halogenated polycyclic aromatic hydrocarbons (e.g. TCDD) to AHR (Zhao, Degroot, Hayashi, He, & Denison, 2010) and allows us to test if TCDD binding to AHR reduces the expression of JAG1. The findings showed that CH-223191 prevented TCDD-stimulated reductions in JAG1 protein (Figure 23B) and supports our hypothesis that TCDD reduces JAG1 by binding to and activating the AHR.

5.4.7. ITE Reduces JAG1 in MCF7 and T47D Cells

TCDD inhibits the growth and proliferation of ER-positive breast cancer cells by antagonizing estrogen signaling (Ohtake, Fujii-Kuriyama, & Kato, 2009). However, there are no reports indicating that TCDD or other AHR ligands modulate the JAG1-Notch pathway in ER-positive breast cancer cells. Considering that a recent report has shown that increases in JAG1 plays a role in tamoxifen resistance in ER-positive breast cancer (Simões, et al., 2015), we further investigated the regulation of JAG1-Notch signaling by AHR in MCF7 and T47D breast cancer cells. To this end, we stimulated MCF7 and T47D cells with the non-toxic endogenous AHR ligand ITE then assayed JAG1 and cleaved-NICD levels by western blot. Cells were treated with vehicle or 10μM ITE every 12 hours for three days. Our rationale for treating cells every 12 hours with 10μM ITE was based on a prior report showing that ITE exerts its maximal

effect at this concentration and that cells must be replenished with ITE every 12 hours due to its rapid metabolism by cells (Cheng, et al., 2015; Henry, Bemis, Henry, Kende, & Gasiewicz, 2006). The western blot results showed that ITE induced downregulation of AHR protein levels, which is consistent with our expectation that ITE is functioning as an AHR ligand in ER-positive breast cancer cells (Ohtake, Fujii-Kuriyama, & Kato, 2009) (Figure 24). As noted earlier, downregulation of AHR protein in response to an AHR ligand is mediated by the ubiquitination of AHR, followed by degradation of AHR by the proteasome (Ma & Baldwin, 2000). The levels of JAG1 protein in MCF7 and T47D cells treated with ITE were significantly lower compared with cells treated with vehicle (Figure 24). Thus, our findings show that TCDD and ITE decrease JAG1 in ER-expressing MCF7 and T47D cells. Next, we analyzed the amount of cleaved-NICD, and the results showed that ITE stimulated increases of cleaved-NICD levels in MCF7 cells compared with the control group (Figure 24). Considering that HES1 is a direct Notch target gene, we questioned whether ITE, by inducing NICD, also promoted the expression of HES1. The findings showed that ITE did indeed increase the levels of HES1 compared with cells treated with vehicle (Figure 24). Collectively, these results suggest that TCDD and ITE decrease JAG1, but are sufficient to activate Notch signaling in ER-positive breast cancer cells. These results are the first to show that the AHR signaling pathway cross-talks with the JAG1-Notch signaling pathway in ER-positive breast cancer cells.

5.4.8. ITE Reduces JAG1 in MDA-MB-231 and MDA-MB-157 Cells

Considering that the JAG1-Notch pathway has been shown to promote the progression of TNBC, we questioned whether the non-toxic AHR ligand ITE reduces JAG1 expression and suppresses Notch signaling in TNBC cells. To this end, we applied vehicle (DMSO) or ITE

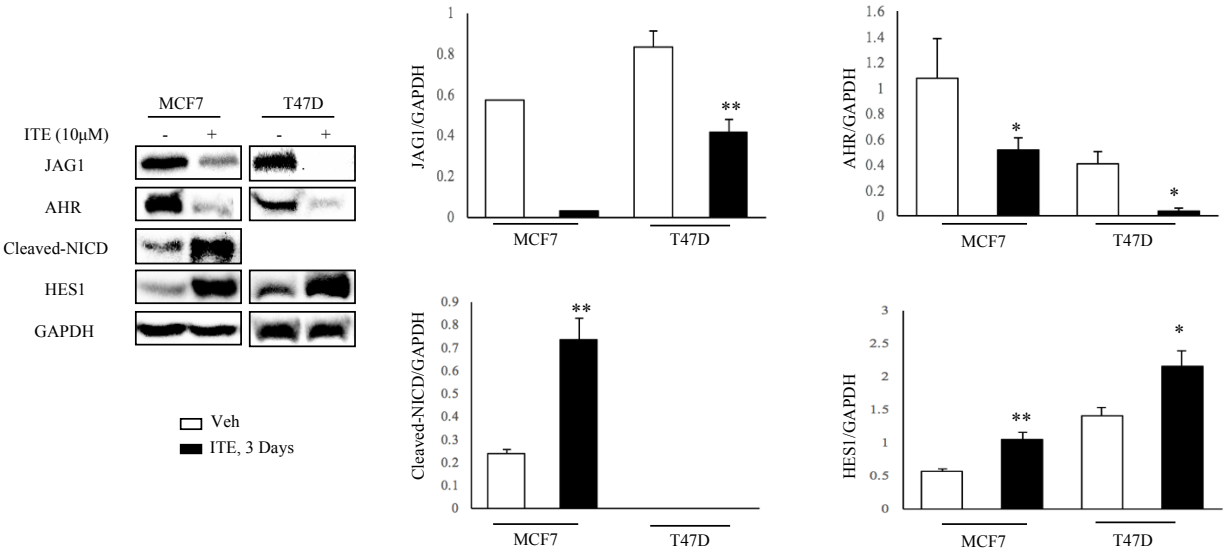


Figure 24. ITE Decreases JAG1 Protein Levels but Activates the Notch Signaling Pathway in ER-positive Breast Cancer Cells

MCF7 and T47D cells were treated with vehicle or 10μM ITE every 12 hours for 3 days. Total cellular protein was isolated and subjected to western blot analysis. Blots were probed with the indicated antibodies. Levels of JAG1, AHR, Cleaved-NICD, and HES1 were normalized to GAPDH. Results shown are the means ± SEM of three independent experiments. Significant changes in expression by ITE are indicated by * $P < 0.05$ or ** $P<0.01$.

(10μM) to MDA-MB-231 cells every 12 hours for 1, 3 and 5 days to investigate whether it reduces JAG1 expression in TNBC cells. We found that the levels of JAG1 protein were 2-fold higher in vehicle-treated cells on day 5 compared with day 1 (Figure 25). Conversely, ITE reduced JAG1 protein on day 1 (by~40%), day 3 (by ~ 50%) and day 5 (by ~ 63%) compared with vehicle-treated controls (Figure 25). Based on these results, we also tested whether ITE treatment would reduce JAG1 mRNA. The mRNA results mirrored the protein findings, showing that JAG1 mRNA levels increased by day 5 compared with day 1 in control cells, and that JAG1 mRNA levels were significantly reduced by ITE on day 3 and day 5 compared with vehicle-treated controls (Figure 25). Collectively, these data indicate that ITE reduces JAG1 mRNA, which may in turn lead to reductions in JAG1 protein. To determine if ITE reduces JAG1 in another TNBC cell line, we applied ITE (10μM) to MDA-MB-157 cells every 12 hours for 3 days and measured the levels of JAG1 protein. We also treated MDA-MB-231 cells as a positive control based on our prior data showing that ITE reduced JAG1 in this TNBC cell line (Figure 25).

Similar to MDA-MB-231 cells, we found that JAG1 expression was also reduced in MDA-MB-157 cells in the ITE-treated group compared to the control group (Figure 26). We also tested whether ITE inhibited Notch signaling by measuring the levels of cleaved-NICD by western blot analysis. The results showed that ITE decreased the amount of cleaved-NICD present in both MDA-MB-231 and MDA-MB-157 cells (Figure 26). To investigate whether the observed reductions in cleaved-NICD levels correlated with a decrease in the levels of a Notch target gene, we probed blots with HES1 antibody. The findings showed that the levels of HES1 were reduced in MDA-MB-231 and MDA-MB-157 breast cancer cells in response to ITE (Figure 26). Based on these findings, we postulate that ITE-stimulated AHR signaling reduces

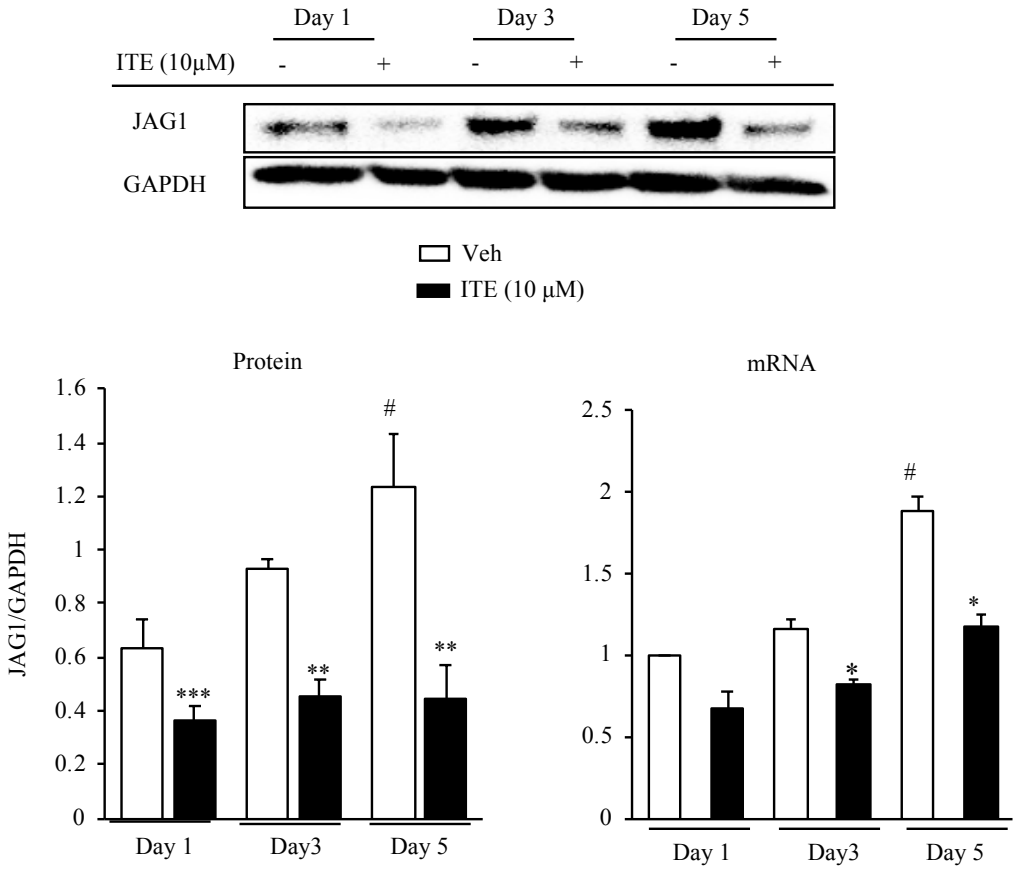


Figure 25. ITE Reduces JAG1 mRNA and Protein in MDA-MB-231 Cells
MDA-MB-231 cells were treated with vehicle or ITE every 12 hours for 1, 3 and 5 days. Total cellular protein was isolated and western blot analysis was performed. Blots were probed with JAG1 and GAPDH. Levels of JAG1 were normalized to GAPDH. Relative mRNA levels of JAG1 normalized to GAPDH loading control was determined by RT-qPCR (as detailed in the Material and Methods). Data shown are the means ± SEM of three independent experiments. Significant increases on Day 5 compared with Day 1 are indicated by $^{\#}$ $P < 0.05$. Significant decreases by ITE are indicated by * $P < 0.05$ or ** $P<0.01$.

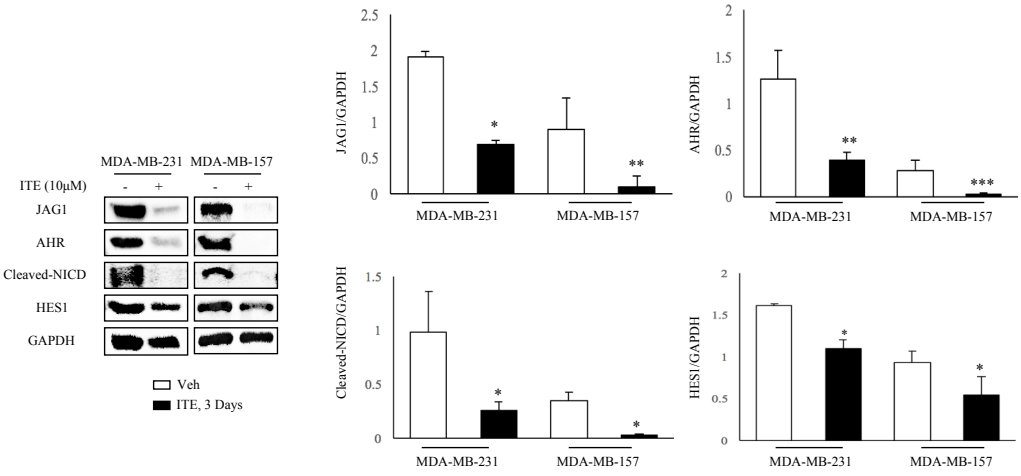


Figure 26. ITE Decreases JAG1 Protein Levels and Inhibits the Notch Signaling Pathway in MDA-MB-231 and MDA-MB-157 Cells

MDA-MB-231 and MDA-MB-157 cells were treated with vehicle or 10μM ITE every 12 hours for 3 days. Total cellular protein was isolated and subjected to western blot analysis. Blots were probed with the indicated antibodies. Levels of JAG1, AHR, Cleaved-NICD, and HES1 were normalized to GAPDH. Results shown are the means ± SEM of three independent experiments. Significant reductions by ITE are indicated by *P < 0.05, **P<0.01, or ***P<0.001.

JAG1, which leads to reductions in cleaved-NICD levels and the expression of the Notch target gene HES1 in TNBC cells.

5.4.9. AHR Mediates ITE-stimulated Reductions in JAG1, Cleaved-NICD, and HES1

Because ITE is an AHR ligand, we postulated that AHR knockdown cells would not respond to ITE. To investigate this postulate, we transfected MDA-MB-231 cells with non-targeting siRNA or AHR-targeting siRNA. Cells were then treated with vehicle or 10μM ITE every 12 hours for 3 days. We found a significant reduction in the levels of JAG1 and AHR protein in the control cells transfected with non-targeting siRNA in response to ITE compared with vehicle (Figure 27). We observed that cells transfected with AHR-targeting siRNA were not responsive to ITE-stimulated reductions in JAG1, indicating that the suppressive effect of ITE on JAG1 expression is transmitted through the AHR (Figure 27). Moreover, the findings showed that siRNA-mediated knockdown of the AHR restored the levels of cleaved-NICD in ITE-treated cells to that observed in control cells treated with vehicle (Figure 27).

Collectively, these results suggest that ITE-activated AHR signaling reduces the expression of JAG1 and inhibits Notch signaling. We reasoned that reductions in JAG1 and cleaved-NICD levels in response to ITE in control cells (transfected with non-targeting siRNA) would lead to reductions in the levels of HES1. We, therefore, probed blots with HES1 antibody, and the findings showed that ITE reduced the levels of HES1 in control cells, but not in the AHR knockdown cells (Figure 27). Collectively, these data suggest that ITE-activated AHR signaling suppresses JAG1-Notch-HES1 signaling in TNBC MDA-MB-231 cells.

As discussed in section 5.3.6., the AHR antagonist CH-223191 preferentially inhibits the binding of TCDD to AHR; however, it does not inhibit the binding of non-halogenated AHR ligands to the AHR (Zhao, Degroot, Hayashi, He, & Denison, 2010). Because ITE is a non-

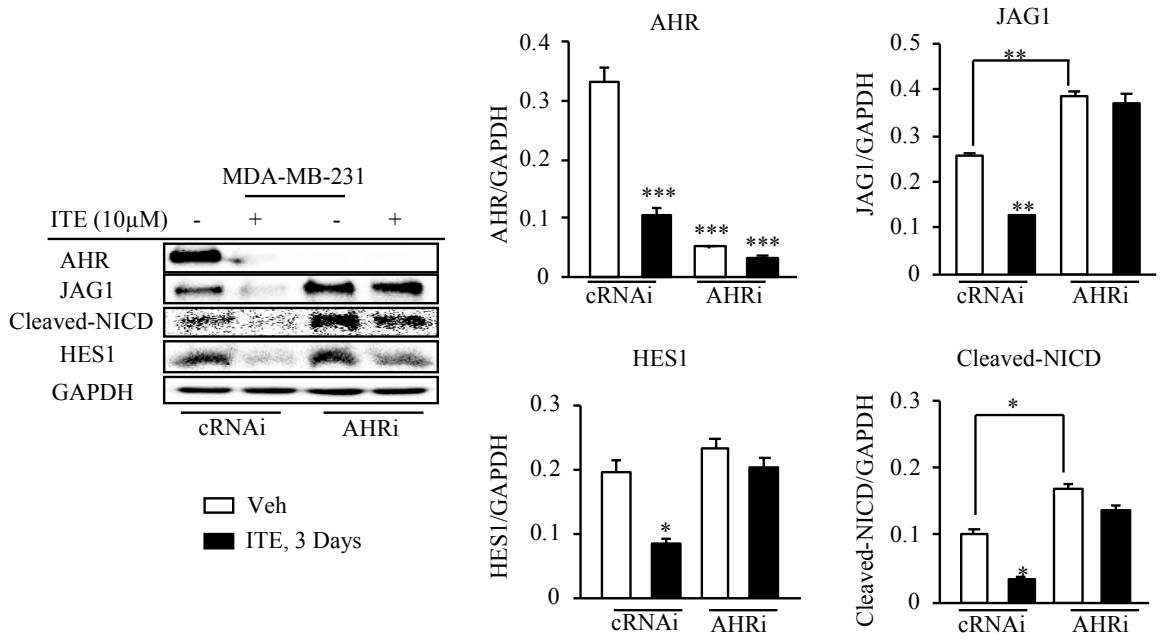


Figure 27. AHR Knockdown Indicates ITE-stimulated Suppression of JAG1 Expression in MDA-MB-231 Cells is AHR-dependent

Control cells were transfected with non-targeting siRNA and AHR knockdown cells were transfected with AHR-targeting siRNA. Forty-eight hours after transfection, cells were treated with vehicle or 10μM ITE every 12 hours for 3 days. Total cellular protein was isolated and subjected to western blot analysis. Blots were probed with the indicated antibodies. Levels of JAG1, AHR, Cleaved-NICD, and HES1 were normalized to GAPDH. Data shown are the means ± SEM of three independent experiments. Significant reductions by TCDD or AHR-targeting siRNA are indicated by *P < 0.05, **P<0.01, or ***P<0.001.

halogenated AHR ligand, we expected that it would not be antagonized by CH-223191. We assessed the levels of JAG1 protein in MDA-MB-231 cells treated with ITE in the absence or presence of CH-223191 and the results showed that this particular AHR antagonist does not block ITE-mediated downregulation of the AHR or JAG1 (Figure 28). This selective-antagonism is consistent with a prior report showing that CH-223191 selectively antagonizes TCDD, but not other types of AHR ligands including ITE (Zhao, Degroot, Hayashi, He, & Denison, 2010). This observed selectivity of CH-223191 for TCDD also suggests that ITE and TCDD reduce JAG1 by activating the AHR through different binding sites on the AHR.

5.4.10. Tranilast Reduces JAG1 in MDA-MB-231 Cells

In addition to investigating the mechanism of how TCDD induces decreases in JAG1 expression, I have addressed how the non-toxic AHR agonist ITE also regulated JAG1 expression in breast cancer cells (Figure 24-28). Therefore, we performed AHR knockdown experiments in MDA-MB-231 cells to determine whether this receptor mediates both TCDD- and ITE-induced downregulation of JAG1 (Figures 23, 27-28). We observed that AHR knockdown cells were unresponsive to both TCDD- and ITE-stimulated reductions in JAG1, indicating that the suppressive effects of TCDD and ITE on JAG1 expression is dependent on AHR activation. We questioned whether there are any other AHR ligands that have been known to promote anti-cancer effects in breast cancer cells that can also reduce expression of JAG1 and have clinical relevance. To answer this question, we treated MDA-MB-231 cells with tranilast to see if it reduces JAG1 expression. Tranilast is a non-toxic AHR ligand that possesses clinical relevance because it is a daily medication that targets inflammatory signaling to treat various allergy symptoms and was shown to inhibit MDA-MB-231 cell growth and metastasis *in vivo* (Prud'homme, et al., 2010). However, the mechanism behind the anti-cancer effects of tranilast

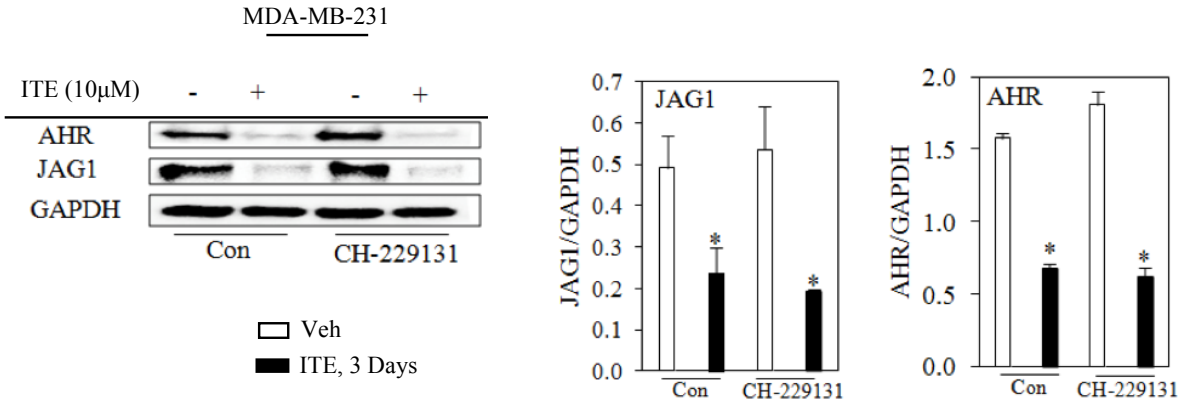


Figure 28. AHR Antagonism via CH-229131 does not Inhibit ITE-stimulated Suppression of JAG1 in MDA-MB-231 Cells

Control cells were treated with vehicle or 10μM ITE every 12 hours for 3 days. CH-223191 groups were treated with 10μM CH-223191 in the absence or presence of 10μM ITE every 12 hours for 3 days. Total cellular protein was isolated and subjected to western blot analysis. Blots were probed with the indicated antibodies. Levels of AHR and JAG1 were normalized to GAPDH. Data shown are the means ± SEM of three independent experiments. Significant reductions by ITE or CH-229131 are indicated by *P < 0.05.

has yet to be determined. We applied tranilast (200μM) to MDA-MB-231 cells every 24 hours for 1, 3 and 5 days to investigate whether it reduces JAG1 expression in TNBC cells. We found that the levels of JAG1 protein were significantly higher in vehicle-treated cells on day 5 compared with day 1 (Figure 29). Conversely, tranilast significantly reduced JAG1 on day 3 (by ~ 65%) and day 5 (by ~ 75%) compared with vehicle-treated controls (Figure 29). This data reveals tranilast as another AHR ligand that promotes anti-cancer effects due to decreasing JAG1 expression and is critically important because it adds clinical relevance to this newly discovered mechanism.

5.4.11. Invasive and Migratory Studies with ITE and JAG1 Knockdown in MDA-MB-231 Cells

Because JAG1 has been reported to be important for the invasive and migratory activity of MDA-MB-231 cells (Shimizu, et al., 2011), we sought to determine whether these MDA-MB-231 processes are inhibited by ITE. To determine the effect of ITE on cell migration, we treated cells with vehicle or ITE every 12 hours for 5 days and then used a pipette tip to make an artificial gap in the confluent cell mass and monitored their capacity to migrate over the gap. We found a significant decrease in the migration of cells treated with ITE compared with those treated with vehicle (40% versus 85% of cells treated with ITE and vehicle, respectively, filled the gap) (Figures 30A and 30B). We also predicted that ITE would suppress the ability of MDA-MB-231 cells to invade through a matrigel-based basement membrane towards a chemoattractant gradient provided by 10% FBS. We first determined whether MDA-MB-231 cells could invade through the matrigel-based basement membrane towards medium supplemented with 10% FBS as the chemoattractant. We observed significant increases (by ~ 155%) in the migration of control cells towards 10% FBS compared with cell culture medium lacking FBS (Figure 30C).

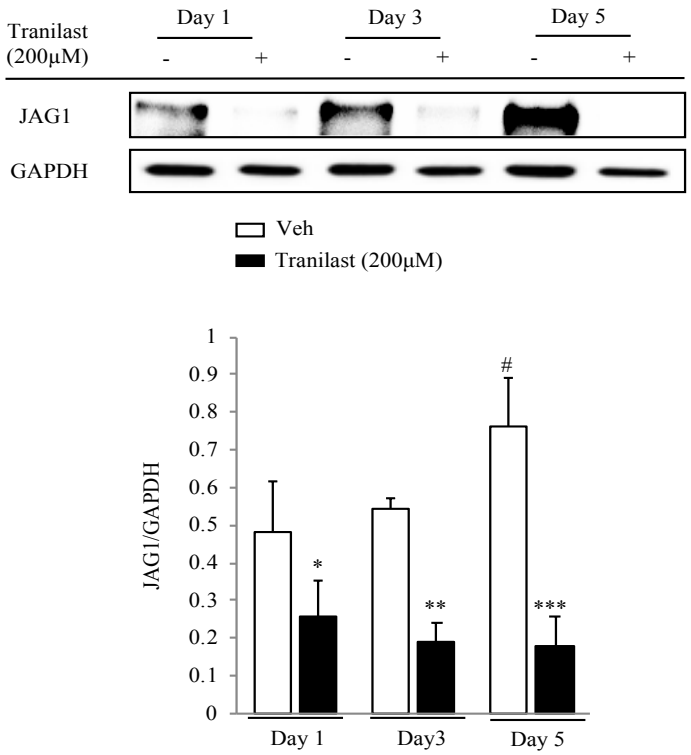


Figure 29. Tranilast Inhibits the Expression of JAG1 in MDA-MB-213 Cells at 1, 3, and 5 Days

MDA-MB-231 cells were treated with vehicle or tranilast every 24 hours for 1, 3 and 5 days. Total cellular protein was isolated and western blot analysis was performed. Blots were probed with JAG1 and GAPDH. Levels of JAG1 were normalized to GAPDH. Data shown are the means ± SEM of three independent experiments. Significant increases on Day 5 compared with Day 1 are indicated by $^{\#}P < 0.05$ Significant decreases by ITE are indicated by *P < 0.05, **P< 0.01, and ***P<0.001.

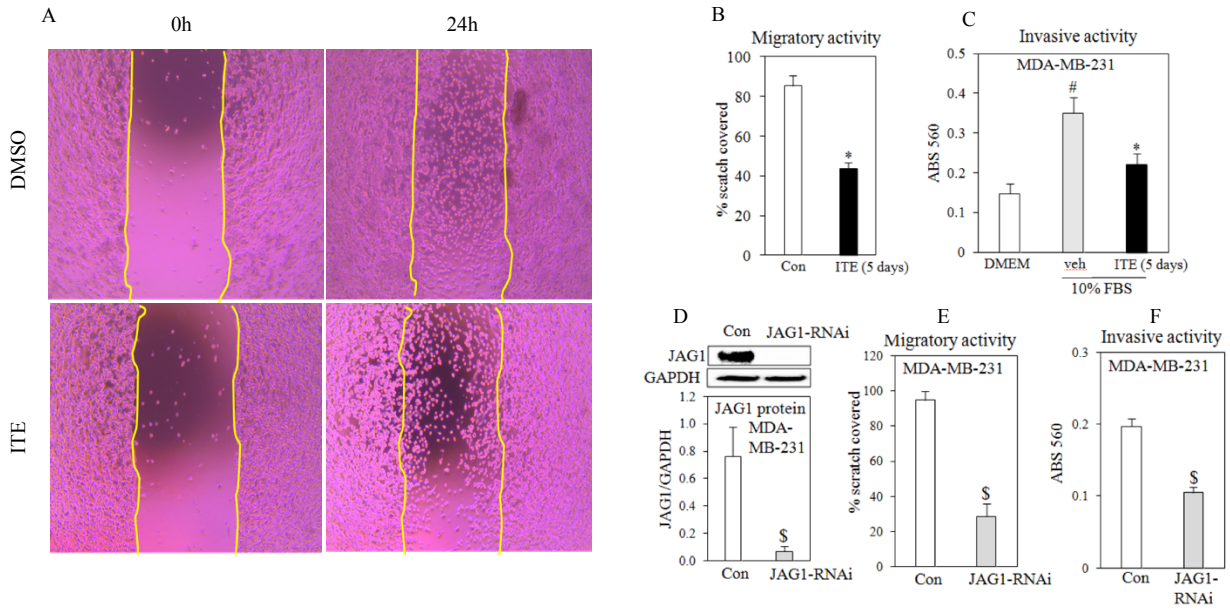


Figure 30. ITE by inhibiting JAG1 Reduces the Migratory and Invasive Activity of MDA-MB-231 Cells

A) Image of the scratch at 0 hours and 24 hours. B-C) Cells were treated with vehicle (DMSO) or 10 μM ITE every 12 h for 5 days prior to the indicated assay. D-F) Control cells were transfected with non-targeting siRNA. JAG1 knockdown cells were transfected with JAG1-targeting siRNA. The indicated assays were performed 48 hours after transfection. D) Total protein was subjected to western blot analysis and blots were probed with indicated antibodies. JAG1 was normalized to GAPDH. B-F) Data shown are means ± SEM of at least 3 independent experiments. Significant increase by 10% FBS is indicated by #$P < 0.05$. Significant reductions by ITE or JAG1-targeting siRNA are indicated by * and \$$P < 0.05$, respectively

Introduction of ITE resulted in a significant decrease (by ~ 40%) in the invasive activity of cells compared with control cells (Figure 30C).

We hypothesized that if ITE inhibits MDA-MB-231 migration by reducing JAG1, then reducing JAG1 with JAG1-targeting siRNA should be sufficient to suppress the invasive and migratory activity of MDA-MB-231 cells. Measuring the levels of JAG1 in MDA-MB-231 cells transfected with JAG1-targeting siRNA showed that JAG1 protein was significantly reduced (by ~ 95%) compared with control cells transfected with non-targeting siRNA, indicating that our JAG1 knockdown approach was valid (Figure 30D). We observed that the cells with JAG1 knockdown had defects that mirrored cells treated with ITE, namely reductions in migratory and invasive activity compared with control cells transfected with non-targeting siRNA (Figures 30E and 30F). Our finding that ITE treatment mimics the loss of migratory and invasive activity of a JAG1 knockdown suggests that ITE affects the behavior of these TNBC cells by reducing JAG1.

5.5. Discussion

The findings of this study have provided the first evidence for crosstalk between ligand-activated AHR signaling and JAG1-Notch signaling pathways in ER-positive and ER-negative breast cancer cells. Considering that the JAG1-Notch pathway has been shown to promote the aggressiveness of breast cancer, our findings suggest that modulating the activity of AHR with AHR ligands could be a novel way to suppress breast cancer progression. This discovery is therefore novel and provides a new perspective into the mechanism(s) by which ligand-activated AHR signaling inhibits breast cancer hallmarks including cell proliferation, invasion and migration (Zhang, et al., 2009; Hall, et al., 2010; Zhang, et al., 2012). We have expanded our previous list of TRGs (137) in MCF7 cells and found that this more extensive list of TRGs (660) is significantly enriched in pathways, which represent new TCDD effects on breast cancer

development (i.e. cell-to-cell communication) (Table 5 and 6). Interestingly, the new TRGs (660) were significantly enriched in cell movement pathways (Table 6 and 8), which supports and extends prior reports showing that TCDD regulates the migratory and invasive activity of breast cancer cells (Zhang, et al., 2012; Hsu, et al., 2007).

It is important to also point out that the ability of ITE to reduce JAG1 expression in breast cancer cells indicates that ITE could be an effective AHR ligand for the treatment of this disease (Figures 24-28). Because the survival of the CSC population is dependent on JAG1-Notch4 signaling (Harrison, et al., 2010; Simões, et al., 2015), it suggests that ITE could target this population of highly tumorigenic cells by targeting JAG1. JAG1 expressed in breast cancer activates Notch signaling in bone, which induces pathways that make bone more amenable to breast cancer cell invasion and therefore metastasis (Sethi, Dai, Winter, & Kang, 2011). Future studies of the role of ITE in these areas of breast cancer research (CSC and metastasis) may lead to new possibilities in the treatment of both ER-positive and ER-negative breast cancer.

This research also acknowledges that AHR activation has a different outcome in ER-positive breast cancer cells, as the Notch pathway was shown to be activated in the presence of TCDD and ITE (Figures 20 and 24). However, the Notch pathway that is activated in ER-positive breast cancer cells may result in a different physiological outcome (i.e. cell morphology) compared to JAG1-dependent Notch activation due to JAG1 having the ability to regulate gene expression independent of the NICD via its intracellular domain (JICD) (Ascano, Beverly, & Capobianco, 2003). Therefore, further research is required to identify unique direct JICD-gene targets that may play a role in the promotion of metastatic behavior and CSC maintenance in breast cancer.

5.5.1. Potential Mechanisms by which TCDD and ITE Differentially Regulate Notch Signaling in ER-negative and ER-positive Breast Cancer Cells

Our results showing that TCDD and ITE increase the levels of cleaved-NICD and HES1 in MCF7 cells suggest that these two AHR ligands activate Notch signaling in ER-expressing breast cancer cells (Figures 20 and 24). In the canonical Notch pathway, the binding of a Notch ligand induces the proteolytic cleavage of the NICD from the Notch receptor and this is mediated by γ-secretase and ADAM (Borggrefe & Liefke, 2012). In mammals, there are five Notch ligands (JAG1, JAG2, DLL1, DLL3 and DLL4) (Chillakuri, et al., 2013). Our finding that ITE and TCDD increase the levels of cleaved-NICD suggests that these two AHR ligands may have increased the expression of a Notch receptor ligand in MCF7 cells. We can rule out JAG1 as a potential Notch ligand that is induced by TCDD and ITE, because our data shows that these two AHR ligands reduce JAG1 expression (Figures 19-29). When considering the remaining four Notch ligands, our RNA-seq analysis identified almost a two-fold increase in the expression of DLL1 in response to the 6 hour 100nM TCDD treatment in MCF7 cells (Table 7 and 11). This suggests that TCDD and ITE may potentially increase the levels of cleaved-NICD by inducing the expression of DLL1, which in turn binds to and activates the Notch1 receptor in MCF7 and T47D cells (Figure 31). However, this observation is speculative. Proving that TCDD and ITE induce Notch signaling in MCF7 and T47D cells by promoting the binding of DLL1 to the Notch receptor will require future experiments. Our findings indicate that ITE and TCDD increase the levels of cleaved-NICD in MCF7 and T47D cells, yet these two ligands decrease the levels of cleaved-NICD in MDA-MB-231 cells (Figures 22-23, 25-28), and ITE decreases levels of cleaved-NICD in MDA-MB-157 cells (Figure 26). Our discovery that TCDD and ITE selectively increased the levels of cleaved-NICD in ER-positive breast cancer cells yet decreased its levels

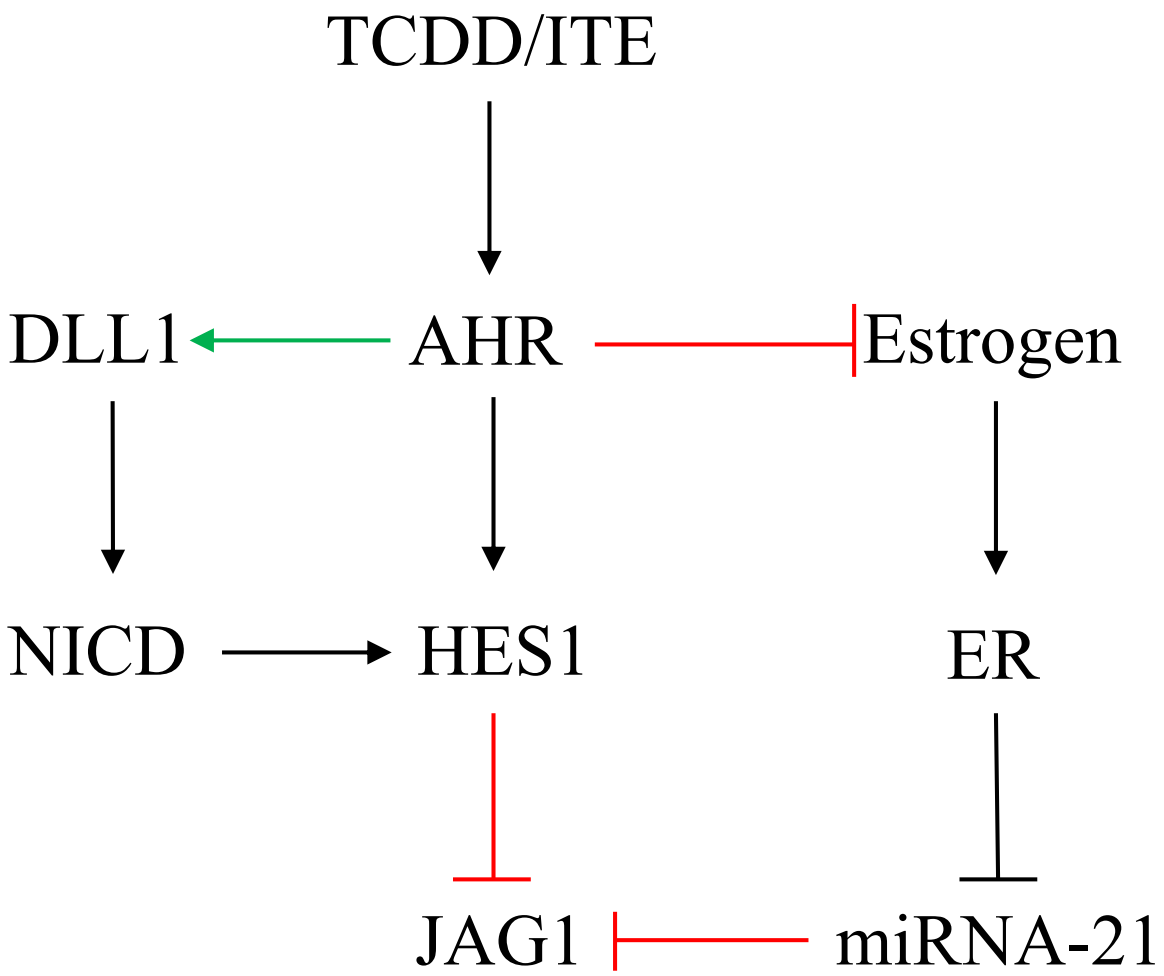


Figure 31. Potential Mechanism(s) that Inhibit JAG1 Expression via AHR Activation in ER-positive Breast Cancer Cells
TCDD = 2,3,7,8-tetrachlorodibenzo-p-dioxin, ITE = 2-(1'H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methylester, AHR = Aryl Hydrocarbon Receptor, DLL1 = Delta-like Ligand 1, ER = Estrogen Receptor, NICD = Notch Intracellular Domain, HES1 = Hairy Enhancer of Split 1, JAG1 = Jagged1, miRNA-21 = microRNA-21. Green Arrows = Increased Expression, Red Arrows = Decreased Expression.

in TNBC cells, suggests that the ER may contribute to the observed increases in cleaved-NICD in response to ligand-activated AHR. The findings of prior reports showing that the AHR and ER crosstalk supports our postulate that the ER may play a role in mediating ITE- and TCDD-stimulated increases in cleaved-NICD levels in ER-positive breast cancer cells.

With regard to TNBC cells, we postulate that the observed decrease in cleaved-NICD levels in response to ITE and TCDD is secondary to the reductions in JAG1 protein. Prior reports have identified that JAG1 is the primary Notch ligand that triggers activation of the Notch1 receptor (leading to increases in cleaved-NICD levels) in TNBC cell lines (Cohen, et al., 2010; Yamamoto, et al., 2013; Dickson, et al., 2007; Reedijk, et al., 2005). We, therefore, hypothesize that TCDD and ITE first downregulate JAG1, which in turn leads to decreases in the activity of Notch signaling and reduces the levels of cleaved-NICD in TNBC cells (MDA-MB-231 and MDA-MB-157). Based on the findings in these prior reports, we propose that the downregulation of cleaved-NICD levels in response to TCDD and ITE in TNBC cells (MDA-MB-231 and MDA-MB-157) is caused by the observed reductions in JAG1 protein levels.

5.5.2. Potential Mechanisms by which TCDD and ITE Reduce JAG1 Expression in ER-negative and ER-positive Breast Cancer Cells

We propose that there are several mechanisms by which TCDD and ITE could reduce the expression of JAG1 in ER-positive and ER-negative breast cancer cells. Regarding ER-expressing breast cancer cells (MCF7 and T47D), the levels of HES1 protein were induced by TCDD and ITE (Figures 20 and 24). Given that HES1 is a transcriptional repressor that has been shown to directly suppress the transcription of JAG1 in other cellular systems (So, et al., 2015; Kobayashi & Kageyama, 2010), it is possible that TCDD and ITE inhibit the expression of JAG1 by increasing the levels of HES1 (Figure 31). However, TCDD and ITE do not increase HES1 in

MDA-MB-231 and MDA-MB-157 cells, indicating that these two AHR ligands do not suppress the expression of JAG1 by increasing the HES1 in these two TNBC cell lines (Figure 26). In addition to HES1, prior reports have shown that miRNA-21 (microRNA-21) inhibits the translation of JAG1 mRNA in MCF7 cells (Selcuklu, Donoghue, Kerin, & Spillane, 2012). Considering that the non-toxic AHR ligand DIM has been reported to increase the expression of miRNA-21 in MCF7 cells (Jin, 2011), it is possible that TCDD and ITE reduces the expression of JAG1 in ER-positive breast cancer cells by increasing the levels of miRNA-21 (Figure 31). The potential for a miRNA-21-based mechanism for suppressing the expression of JAG1 in response to ligand-activated AHR is more likely to occur in ER-positive breast cancer cells and not TNBC cells, given that prior reports have shown that this particular microRNA is not inducible in TNBC cells (i.e., MDA-MD-231 and MDA-MB-468) (Selcuklu, Donoghue, Kerin, & Spillane, 2012; Jin, 2011).

For reasons detailed in the previous paragraph, we postulate that increases in HES1 or miRNA-21 in response to TCDD or ITE could induce the observed downregulation of JAG1 in ER-positive, but not TNBC cells. We hypothesize that AHR-mediated suppression of NF-κB is the primary mechanism by which ITE and TCDD suppress the transcription of JAG1 in TNBC cells (MDA-MB-231 and MDA-MB-157) (Figure 32). NF-κB is a transcription factor that is more highly expressed and active in TNBC compared with ER-positive breast cancer (Yamamoto, et al., 2013; Yamaguchi, et al., 2009). The higher activity and expression of NF-κB in TNBC cells is attributed to constitutive expression of pro-inflammatory cytokines (i.e., IL-6 and TNFα), which act through their cell surface receptors to induce signaling that increases the levels and activity of NF-κB (Figure 13). The NF-κB family is composed of five members p50, p52, RelA, RelB, and c-Rel, which promote JAG1 expression (Yamamoto, et al., 2013).

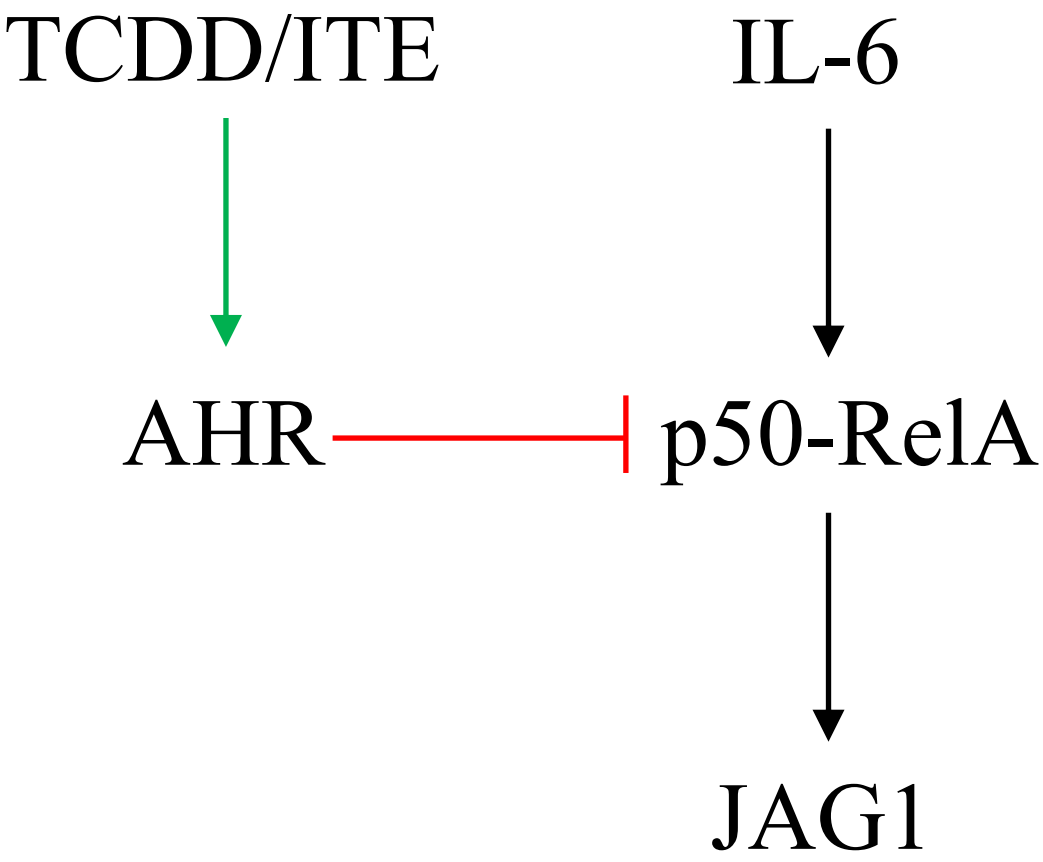


Figure 32. Potential Mechanism that Inhibits JAG1 Expression via AHR Activation in ER-negative Breast Cancer Cells
TCDD = 2,3,7,8-tetrachlorodibenzo-p-dioxin, ITE = 2-(1'H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methylester, AHR = Aryl Hydrocarbon Receptor, IL-6 = Interleukin-6, RelA = V-Rel Avian Reticuloendotheliosis Viral Oncogene Homolog A. Green Arrows = Increased Expression, Red Arrows = Decreased Expression.

The primary NF-κB subunit that has been reported to transcriptionally activate JAG1 is RelA (Bash, et al., 1999; Johnston, Dong, & Hughes, 2009). Furthermore, RelA was identified to specifically increase the transcription of JAG1 and not the other four Notch ligands (i.e., JAG2, DLL1, DLL3 and DLL4) (Yamamoto, et al., 2013). Prior reports have shown that the AHR signaling pathway extensively crosstalks with the NF-κB pathway to influence target gene expression (Tian, 2009; Beischlag, Luis Morales, Hollingshead, & Perdew, 2008; Kim, et al., 2000; Salisbury & Sulentic, 2015) (Figure 13). Notably, TCDD-activated AHR signaling inhibits the binding of RelA to NF-κB binding sites in target genes (Tian, 2009; Salisbury & Sulentic, 2015) (Figure 13). Based on the findings of these prior reports, we hypothesize that ITE or TCDD-activated AHR signaling inhibits the binding of RelA to NF-κB binding sites on the JAG1 promoter, and reduces the transcription and expression of JAG1 in TNBC cells (Figure 32). Proving that ligand-activated AHR signaling suppresses RelA binding to the promoter of JAG1 can be addressed in future studies.

5.5.3. Potential Mechanisms by which ITE Inhibits the Migratory and Invasive Activity of MDA-MB-231 Cells

Our findings show that ITE inhibits the migratory and cell invasive activity of MDA-MB-231 cells (Figures 29A-29C). Prior reports have shown that JAG1 and cleaved-NICD increase the movement and invasive activity of MDA-MB-231 cells (Leong, et al., 2007; Shimizu, et al., 2011). Our data indicates that ITE reduced the levels of JAG1 (Figures 25-28) and cleaved-NICD (Figures 26-27) in TNBC cells. We, therefore, propose that ITE inhibits the migratory and invasive activity of MDA-MB-231 cells by decreasing the levels of JAG1 and cleaved-NICD (Figure 29). Prior reports have provided insight into the mechanisms by which JAG1 and cleaved-NICD promote the migratory and invasive activity of TNBC cells using the

MDA-MB-231 cell model (Shimizu, et al., 2011). The secreted serine protease, μPA, converts plasminogen to plasmin, and it has been shown to be a marker of breast cancer recurrence, high metastatic risk, and poor breast cancer prognosis (Shimizu, et al., 2011). Shimizu et al. demonstrated that siRNA-mediated knockdown of JAG1 or Notch1 caused reductions in the expression of μPA in three TNBC cell lines, including MDA-MB-231 cells (Shimizu, et al., 2011). The authors conducted further work and showed that reducing the expression of JAG1 or μPA was sufficient to suppress the migratory and invasive activity of MDA-MB-231 cells (Shimizu, et al., 2011). Collectively, Shimizu et al demonstrated that JAG1-mediated Notch signaling induced the transcription of μPA upon the NICD binding to the promoter region of the μPA gene, which in turn promoted the invasive and migratory activity of MDA-MB-231 cells (Shimizu, et al., 2011). While our findings show that ITE reduces the levels of JAG1 and cleaved-NICD in TNBC cells, a future study will be required to investigate whether ligand-activated AHR signaling also reduces the expression of μPA in MDA-MB-231 cells.

In addition to inducing the expression of μPA, JAG1 and cleaved-NICD have been reported to increase the transcription and expression of a Snail Family of Transcriptional Repressor (SNAI) member (Leong, et al., 2007). The SNAI2 (Slug) gene encodes a transcriptional repressor that upon binding to E-box motifs suppresses the transcription and expression of E-cadherin (Bolós, et al., 2003). The suppression of E-cadherin induces EMT and promotes the mesenchymal phenotype, which is more migratory and invasive compared with the properties of the epithelial phenotype (Bolós, et al., 2003). Thus, JAG1 and the NICD have been proposed to increase the migratory and invasive activity of TNBC cells by increasing the expression of Slug, which in turn downregulates E-cadherin and promotes EMT (Niessen, et al., 2008). Considering the findings of these prior reports, it is possible that ITE by reducing the

levels of JAG1 and the NICD also reduces the expression of Slug and suppresses EMT in TNBC cells. Recent studies have also suggested that Notch signaling induces a specialized type of EMT during normal heart development and that Notch up-regulates SNAI1 (Snail) in endothelial cells to promote mesenchymal transformation (Timmerman, et al., 2004; Noseda, et al., 2004). Whether ITE downregulates Slug or Snail in TNBC cells can be addressed in future studies.

5.6. Conflicts of Interest

The authors declare that they have no competing interests.

5.7. Acknowledgements

This work was supported by a grant from the West Virginia - Institutional Development Award Program (IDeA) Networks of Biomedical Research Excellence (WV-INBRE) grant (P20GM103434 from NIGMS) and support from the Marshall University Genomics and Bioinformatics Core. The opinions in this paper are the authors and not those of the NIH.

CHAPTER 6

SNAIL: AN ESSENTIAL REGULATOR FOR EMT

The SNAI family of zinc-finger transcription factors consist of Snail, Slug and SNAI3 (Smuc), which all share an evolutionarily conserved role in mesoderm formation in vertebrates (Nieto, 2002). They possess a highly conserved carboxy-terminal region that contain 4-6 acetylene-type zinc fingers, which regulate sequence-specific interactions within DNA promoters containing a CAGGTG E-box sequence (Wu & Zhou, 2010). Snail expression in both vertebrate and Drosophila suppress gene expression through the interaction with the co-repressor C-terminal binding protein (CtBP) and also by directly promoting repressor complex formation (Nieto, 2002). Peinado et al. was able to demonstrate that Snail interacted with co-repressor complex Histone Deacetylase Complex Subunit Sin3a (SIN3A), histone deactylase 1 (HDAC1), and histone deactylase 2 (HDAC2) in order to inhibit E-cadherin expression (Peinado, Ballestar, Esteller, & Cano, 2004).

Snail employs global effects on the genome in epithelial cells and by altering the gene expression profile. Therefore, it is involved in regulating EMT (Kalluri & Weinberg, 2009), cell survival (Emadi Baygi, Soheili, Schmitz, Sameie, & Schulz, 2010), apoptosis (Kurrey, et al., 2009), cell polarity (Moreno-Bueno, Portillo, & Cano, 2008) and stem cell-like properties (Kurrey, et al., 2009). The regulatory and functional roles of Snail emerged as one of the hottest topics in medicine these past few years in cancer biology. This chapter will focus on the regulation of Snail and discuss its functions in EMT and stem cells, as well as how our lab shows that TCDD and ITE regulate Snail in an AHR-dependent manner.

6.1. Regulation of Snail

Snail has been identified as a highly unstable protein and is regulated by both protein stability and cellular location. The expression of Snail is regulated by an integrated and complex signaling network at the transcriptional and post-transcriptional level. This network includes integrin-linked kinase (ILK), PI3K, MAPKs, GSK-3β, and NF-κB pathways (De Craene, van Roy, & Berx, 2005; Wu, Y. et al., 2009). Fibroblast growth factor (FGF) and EGF signaling induces Snail expression by suppressing the activity of GSK-3β (Wu & Zhou, 2010).

Many signaling pathways that are involved in embryonic development also regulate Snail expression. One example is the TGFβ signaling pathway, which induces Snail expression via Smad binding directly to the Snail promoter in hepatocytes, epithelial and mesothelial cells (Xu, Lamouille, & Derynck, 2009). Additionally, Notch signaling utilizes two distinct mechanisms that regulate Snail expression synergistically by recruiting the NICD to the Snail promoter and by NICD-HIF1α promoting expression of lysyl oxidase (LOX), a known stabilizer of Snail (Sahlgren, Gustafsson, Jin, Poellinger, & Lendahl, 2008; Peinado, et al., 2005). The Wnt signaling pathway also stabilizes Snail by suppressing GSK-3β activity (Yook, Li, Ota, Fearon, & Weiss, 2005).

Snail expression can also be regulated by the NF-κB pathway via transcriptional and post-transcriptional mechanisms. GSK-3β inhibition stimulates the transcription of Snail by activating the NF-κB pathway (Bachelder, Yoon, Franci, de Herreros, & Mercurio, 2005). Post-transcriptionally, TNFα is the major signaling pathway that induces Snail stabilization. TNFα-NF-κB-stabilized Snail is mediated by the transcriptional induction of casein kinase-β (CSN2), which results in the inhibition of phosphorylation and ubiquitylation of Snail by GSK-3β (Wu, Y., et al. 2009).

6.2. Snail, EMT, and Stem Cells

EMT is a profound event for large-scale cell movement during morphogenesis at the time of embryonic development (Wang, Shi, Chai, Ying, & Zhou, 2013). During this process, epithelial cells detach from neighboring cells and gain mesenchymal properties, which enables them to break through the basement membrane that separates different tissues within the embryo (Kalluri & Weinberg, 2009). One of the hallmarks of EMT is the functional loss of E-cadherin, which is thought to be metastatic suppressor during tumor growth and progression (Hanahan & Weinberg, 2011). Snail is a prominent inducer of EMT by strongly repressing E-cadherin expression (Barrallo-Gimeno & Nieto, 2005).

Because a similar process occurs at the invasive front of metastatic cancer, it has been proposed that tumor cells exploit the developmental EMT program for their metastatic dissemination. Metastasis is responsible for a majority of cancer patient deaths and is divided into a series of steps, including detachment of tumor cells from the primary tumor, invasion, intravasation, anoikis-resistance, extravasation, and secondary-site colonization (Pantel & Brakenhoff, 2004). EMT is involved in the metastatic cascade of many solid tumors and entails the molecular reprogramming and phenotypic changes that characterize the conversion of immobile cancer epithelial cells to motile mesenchymal cells. Therefore, expression of Snail positively correlates with tumor grade, recurrence, metastasis, and poor prognosis in various cancer types (Peinado, Olmeda, & Cano, 2007; Hemavathy, Ashraf, & Ip, 2000; Moody, et al., 2005; Chen, et al., 2010).

From a physiological stand-point, stem cells are the basis for tissue homeostasis in the adult organism (Fuchs, Tumbar, & Guasch, 2004). Recent evidence has shown that Snail plays a role in the preservation of stem cell function. de Frutos et al was able to demonstrate that Snail

plays a fundamental role in controlling bone mass and bone homeostasis by acting as a repressor of RUNX2 and Vitamin D Receptor (VDR) transcription (de Frutos, et al., 2009). The expression of Snail is tightly regulated in bone development and its activity on osteoblasts regulates bone cell differentiation in order to ensure proper bone remodeling (Wu & Zhou, 2010). Additionally, Snail also regulates genes that are involved in neural stem cell self-renewal and multi-potency (Southall & Brand, 2009). These observations identify that Snail is an important factor to the preservation of stem cell function and maintenance.

6.3. TCDD and ITE Regulate Snail in an AHR-dependent Manner

Knowing that JAG1-dependent Notch signaling promotes expression of various target genes that are required to promote cancer growth at various stages of tumor progression, we questioned if whether the target genes downstream are also affected by ITE and TCDD treatments in breast cancer cells. Therefore, we measured the changes of Snail, a down-stream target of JAG1-Notch1 signaling that is critical for promoting EMT by suppressing the expression of E-cadherin (Li, Masiero, Banham, & Harris, 2014).

When T47D and MDA-MB-231 cells were treated with 10nM TCDD, our data indicated that TCDD was able to significantly decrease Snail expression after 24 hours (Figure 33). In the case of ITE, we treated T47D, MDA-MB- 231, and MDA-MB-157 cells with 10μM ITE for 3 days to determine if there were any changes in Snail expression and observed decreases in Snail in all three cell lines (Figure 33), signifying that the regulation of Snail via activation of the AHR is independent of the ER.

In order to determine if regulation of Snail was AHR-dependent, MDA-MB-231 cells transfected with non-targeting siRNA or AHR-targeting siRNA were treated with vehicle or 10 μM ITE for 3 days. We found a significant reduction in the levels of Snail protein in the

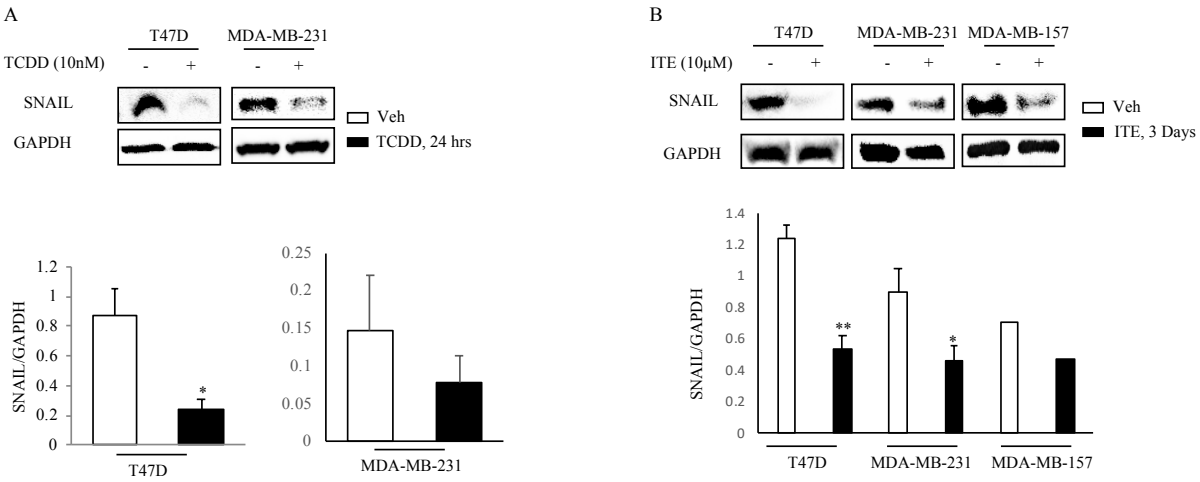


Figure 33. Activation of AHR via TCDD and ITE Inhibits Snail Expression in Breast Cancer Cells

A) T47D and MDA-MB-231 cells were treated with vehicle or 10 nM TCDD for 24 h. B) T47D, MDA-MB-231, and MDA-MB-157 cells were treated with vehicle or 10μM ITE every 12 h for 3 days. Total cellular protein was isolated and subjected to western blot analysis. Blots were probed with the indicated antibodies. Levels of Snail were normalized to GAPDH. Results shown are the means ± SEM of three independent experiments. Significant reductions by ITE are indicated by *P < 0.05 or **P<0.01.

control cells treated with ITE compared with cells treated with vehicle (Figure 34). Finding that ITE reduces the levels of Snail protein in control cells is consistent with our prediction that ITE is functioning as an AHR ligand in MDA-MB-231 cells. We observed that AHR knockdown cells were not responsive to ITE-stimulated reductions in Snail, indicating that the suppressive effect of ITE on Snail expression is transmitted through the AHR (Figure 34). Collectively, our data suggests that various AHR ligands that have been known to promote anti-cancer effects is potentially achieved through a common mechanism of reducing JAG1 expression. This in turn would expect to decrease the expression of genes that are targets of JAG1-dependent signaling, which are required for cancer progression.

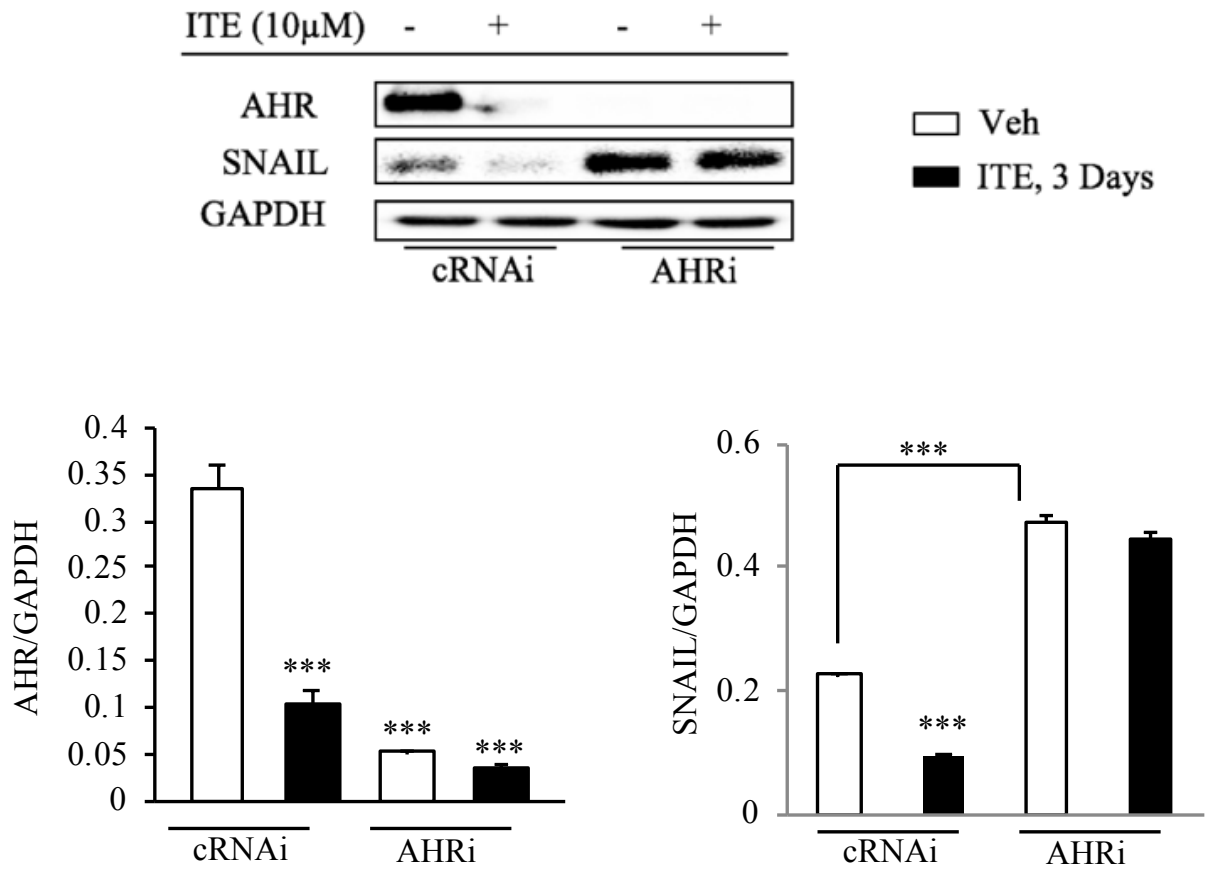


Figure 34. AHR Knockdown Indicates ITE-stimulated Suppression of Snail Expression in MDA-MB-231 Cells is AHR-dependent

Control cells were transfected with non-targeting siRNA and AHR knockdown cells were transfected with AHR-targeting siRNA. Forty-eight hours after transfection, cells were treated with vehicle or 10μM ITE every 12 hours for 3 days. Total cellular protein was isolated and subjected to western blot analysis. Blots were probed with the indicated antibodies. Levels of Snail and AHR were normalized to GAPDH. Data shown are the means ± SEM of three independent experiments. Significant reductions by ITE or AHR-targeting siRNA are indicated by ***P<0.001.

CHAPTER 7

DISCUSSION AND FUTURE DIRECTIONS

7.1. Discussion

Towards the end of my research project, AHR signaling became even more fascinating compared to when I first began studying it over three years ago. With AHR biology being so diverse in its ability to bind various ligands, the endogenous roles seem to reveal themselves slowly and branch out into a variety of cellular functions. In the realm of toxicology, the AHR is one of the most commonly studied receptors, given the breadth of compounds which work through AHR signaling to exert their effects. Even though the AHR has been studied extensively in response to over 400 exogenous ligands, which include environmental pollutants/toxicants, various drugs, and phytochemicals, there is still much to learn about endogenous AHR cellular signaling. It is important to note that the AHR is an evolutionarily conserved protein, which has played a vital role for allowing researchers to make great strides in understanding the endogenous function of AHR. Moreover, researchers were also able to help characterize endogenous AHR ligands that are produced by the microflora present in the body, based on diet composition (Denison, Soshilov, He, DeGroot, & Zhao, 2011). While AHR research has shifted from toxicology-based to a more cancer-based emphasis (Safe, Cheng, & Jin, 2017), it is clear there is still much to learn regarding AHR functionality in both normal and cancer cell physiology.

Our research objective was simple as well as diverse, which was to reveal a new tumor suppressing role for the AHR in breast cancer by hypothesizing that non-toxic AHR ligands can induce anti-cancer effects by regulating the expression of JAG1 and the Notch signaling pathway. Since that has been accomplished, we can continue to approach this objective from

alternate perspectives, as will be detailed later in this chapter. First, our findings show that TCDD and ITE reduce JAG1 in both ER-positive (MCF7 and T47D) and ER-negative (MDA-MB-231 and MDA-MB157) breast cancer cells as a novel discovery and provides a new insight into the mechanism by which ligand-activated AHR signaling inhibits cancer hallmarks including cell invasion and migration (Figures 19-29). However, our findings also show that the AHR acts as a Notch signaling modulator, which is potentiated in a cell-type dependent manner. This is due to the AHR activating the Notch signaling pathway in ER-positive cells (Figures 20 and 24) but inhibiting it in ER-negative cells (Figures 22, 26-27). Additionally, we were able to characterize cell-cell communication and cell movement as new bio-functional roles for AHR through RNA-seq analysis (Table 6). Therefore, future work involving the identification of downstream target genes that are unique to JAG1-dependent Notch signaling and that are also regulated by AHR activation would increase the evidence for the tumor-suppressing roles of the AHR. The following sections will cover the therapeutic implications for targeting the AHR in both ER-positive and ER-negative breast cancer cells.

7.1.1. Therapeutic Implications for Targeting ER-positive Breast Cancer via AHR Activation

It is important for us to consider the potential therapeutic implications for targeting the AHR through the use of non-toxic agonists for the treatment of ER-positive breast cancer. It has been demonstrated that the protective effects of AHR ligands on tumor growth are related to the ability of the receptor to antagonize ER signaling (Murray, Patterson, & Perdew, 2014). The functional outcomes of the antagonistic AHR-ER crosstalk are apparent in both *in vitro* and *in vivo* models in which TCDD was shown to completely reverse the proliferative effects of estrogen mediated signaling (Weng, Tsai, Kulp, & Chen, 2008; McDougal, Wormke, Calvin, &

Safe, 2001). In addition to the antagonistic effects on ER signaling, the AHR also regulates key processes required for breast cancer cell growth (McDougal, Wilson, & Safe, 1997), cell cycle control (Barhoover, Hall, Greenlee, & Thomas, 2010), chemokine signaling (Hsu, et al., 2007), and cell migration (Hall, et al., 2010).

There are some cases that argue activation of the AHR promotes breast cancer progression and that inhibiting AHR activity is the best route for the treatment of ER-positive breast cancer. Dubrovska et al. demonstrated tamoxifen-resistant MCF7 cells maintain the CSC population through the C-X-C motif chemokine receptor 4 (CXCR4), which resulted in the activation of AHR-dependent gene transcription (Dubrovska, et al., 2012). This research proposed that inhibition of CXCR4 or AHR (via antagonists) specifically targets the CSC population and could be beneficial for the treatment of tamoxifen-resistant breast cancer. However, it is uncertain if AHR activation occurs in a ligand-dependent manner in this particular case, as the role of the AHR in facilitating cell cycle progression is primarily manifested in the absence of an exogenous ligand because treatment with AHR agonists inhibits cell cycle progression (Gomez-Duran, et al., 2009; Greenlee, Hushka, & Hushka, 2001; Elferink, Ge, & Levine, 2001). It is therefore important to consider the possibility that there is a genuine ligand-independent AHR activation mechanism as it was demonstrated by Ikuta et al, who showed that tumor cells with high levels of the AHR undergo dynamic nucleocytoplasmic shuttling (Ikuta, et al., 2000). Dynamic nucleocytoplasmic shuttling could lead to AHR and ARNT heterodimerization in the absence of ligand and present a different physiological outcome compared to ligand-activated AHR signaling. This mechanism could also potentially occur in the CSC population of ER-positive breast cancer cells, where the AHR promotes pro-tumorigenic characteristics in a ligand independent manner (Oesch-Bartlomowicz, et al., 2005). However, our

lab measured gene expression with cancer profiling gene arrays that revealed TCDD-treated MCF7 breast cancer cells decreased the mRNA expression of CXCR4, signifying another addition to the tumor-suppressing role for ligand activated AHR signaling in MCF7 cells (Raw reads and processed RNA-seq data were deposited in the Gene Expression Omnibus (GEO) at the National Center for Biotechnology Information and are accessible via accession number GSE98515). Hall et al. also demonstrated that TCDD inhibited expression of CXCR4 in both ER-positive and ER-negative breast cancer cells (Hall, et al., 2010). Interestingly, Chiaramonte et al. demonstrated that Notch activation was able to promote both multiple myeloma and ovarian cancer growth and metastasis by promoting the expression of CXCR4, making the Notch signaling pathway a viable target for treatment in these particular cancers (Chiaramonte, et al., 2015). Based on our data and current literature, it is possible that AHR activation inhibits JAG1 expression, resulting in the decrease of CXCR4 mRNA expression as shown by our RNA-seq analysis. Therefore, it is safe to postulate that even though both ITE and TCDD activated the Notch pathway in ER-positive breast cancer by increasing cleaved-NICD and HES1 levels, the decreased expression of JAG1 and CXCR4 indicates that JAG1-dependent Notch signaling may be responsible for the regulation of CXCR4. However, further studies must be conducted to fully elucidate this mechanism.

A study conducted by Al-Dhfyan et al. provides another example on how AHR activation mediates the expansion of the BCSC population in MCF7 cells. This extensive study provided the first strong evidence that activation of the AHR-CYP1A1 pathway via TCDD and 7,12-dimethylbenz(a)anthracene (DMBA) promoted CSC development, maintenance, self-renewal, and chemo-resistance through inhibition of phosphatase and tensin homolog (PTEN) and activation of β-catenin and Akt-pathways (Al-Dhfyan, Alhoshani, & Korashy, 2017). Moreover,

they showed that inhibition of the AHR via α-naphthoflavone sensitized CSCs to chemotherapy by increasing the percentage of apoptotic CSCs in response to doxorubicin. However, even though this study provides mechanistic insight into the relationship between AHR activation and the CSC population from a toxicological stand-point, it does not establish appropriate clinical relevance for antagonizing AHR for the treatment of ER-positive breast cancer. This study utilized only highly stable carcinogenic compounds to determine functional relevance of AHR activation on BCSCs as a means to promote AHR antagonism as a viable form of treatment. Additionally, this study did not provide any evidence if this same outcome occurs with known non-carcinogenic AHR agonists that have been demonstrated to promote inhibitory effects on the CSC population and possess true clinical relevance. AHR agonists such as tranilast (Prud'homme, et al., 2010), IC3 (Weng, Tsai, Kulp, & Chen, 2008), and DIM (Jin, 2011) were shown to effectively inhibit the growth of both ER-positive and ER-negative breast cancer similar to that of TCDD with none of the adverse effects. Moreover, their data regarding TCDD treatments in MCF7 cells does not align with our data, as they showed that TCDD did not increase the nuclear translocation of the NICD, hence why they claim the Wnt signaling pathway is involved in CSC development, maintenance, and self-renewal. This can potentially be due to the differences in our timed treatments as they treated MFC7 cells with TCDD for 3 days, which may result in identifying down-stream effects of TCDD treatments rather than initial changes in gene expression. Finally, and most importantly, this study looked at different functional outcomes compared to our research as they studied AHR-regulated changes in mammosphere formation and chemo-resistance while we observed changes in metastatic behavior involving invasion and migration. Overall, what these discrepancies dictate is that the activation of the AHR in the context of promoting the CSC phenotype in ER-positive breast cancer may require a

process that balances the apparent advantage of modulating AHR activity for invasion, motility, and colonization with the need for growth and proliferation. Unfortunately, the role of the AHR in cellular physiology suffers from the toxicological origins of the receptor. In reality, the AHR should be viewed in the same realm as other cellular receptors (i.e. ER, AR, and PR) with a normal physiological role that can be disrupted by xenobiotic chemicals rather than a receptor that evolved primarily as a xenobiotic sensor.

Despite the discrepancies present with AHR activation regarding the promotion of cell proliferation and stem-cell maintenance, our lab was able to show AHR activation prevents cancer promoting bio-functions, such as EMT and the metastatic process (i.e. invasion and migration, see Figure 29). These anti-cancer functions are easily postulated due to our discovery that both TCDD and ITE inhibit JAG1 expression in MCF7 cells, a vital component for the promotion of metastasis, drug-resistance, and tumor recurrence (Li, Masiero, Banham, & Harris, 2014). Our hypothesis that TCDD inhibits these invasive and metastatic features in breast cancer cells correlates with a study conducted by Hall et al., that demonstrated breast cancer cells treated with exogenous AHR agonists (TCDD and DIM) significantly inhibited cell invasiveness and motility by Boyden chamber assay and inhibited colony formation, in ER-positive cells (Hall, et al., 2010). This is because they observed that AHR activation reduced the number of invaded cells by 70% in MCF7, SKBR3, and ZR-75-1 breast cancer cell lines. In correlation with this functional outcome, TCDD was able to significantly repress CXCR4 and matrix metallopeptidase 9 (MMP9), two prominent genes heavily associated with metastasis (Hao, et al., 2007). This observation not only correlates with our research, but also with a study conducted by Dai et al, that demonstrated knockdown of JAG1 via siRNA resulted in decreased expression of MMP9 in colorectal cancer cells, resulting in decreased invasion (Dai, et al.,

2014). Additionally, knockdown of AHR via siRNA and the use of the AHR antagonist α-naphthoflavone demonstrated that these anti-invasive outcomes were AHR-dependent (Hao, et al., 2007). Together these studies suggest that the inhibition of motility and invasiveness by exogenous AHR agonists are receptor-mediated and that endogenous AHR activity promotes an anti-cancer role through inhibiting the metastatic process. Our discovery that the decreased expression of JAG1 via TCDD and ITE in MCF7 cells could help elucidate the mechanism behind the anti-metastatic effects of AHR activation.

Ultimately, the use of the non-toxic AHR agonists such as ITE in the presence of anti-estrogenic treatments such as tamoxifen may provide a synergistic effect in the treatment of ER-positive breast cancer, especially drug-resistant breast cancer. The therapeutic strategy combining AHR agonists and anti-estrogenic therapies is valid and logical, based on the study conducted by Darakhshan & Ghanbar, where the combination of tranilast (a potent AHR agonist which will be covered later in this chapter) and tamoxifen induced anti-tumorigenic effects in a synergistic manner in MCF7 breast cancer cells (Darakhshan & Ghanbar, 2013).

7.1.2. Therapeutic Implications for Targeting ER-negative Breast Cancer via AHR Activation

Some early stage and most later stage mammary tumors are ER-negative and patients with ER-negative breast cancer do not respond well to endocrine therapy; successful adjuvant chemotherapy requires the use of more highly cytotoxic agents commonly used to treat other endocrine-independent tumors. These agents generally target some aspect of nuclear function (i.e. DNA-chelation) or modulate microtubule formation/breakdown and include compounds such as doxorubicin, cyclophosphamide, gemcitabine, taxanes, and capecitabine, a precursor of 5-flurouracil (5-FU) (Ismail-Khan & Bui, 2010). Evidence and observations from multiple

studies on the use of AHR agonists for the treatment in ER-negative breast cancers possesses less discrepancy and controversy compared to that of ER-positive breast cancer. These observations may be due to the increasing evidence that the AHR plays a role in the inhibition of the metastatic and invasive cellular processes, which are much more prominent in the ER-negative breast cancer subtype (Hanieh, 2015; Hall, et al., 2010; Prud'homme, et al., 2010). These observations stem from various extensive studies that show that treatment with exogenous AHR agonists significantly inhibited cell invasiveness and motility in the Boyden chamber assay and inhibited colony formation in soft agar regardless of ER, PR, or HER2 status (Hall, et al., 2010; Meng, et al., 2000; Darakhshan & Ghanbar, 2013). This observation further signifies that the anti-cancer mechanism behind AHR activation is not limited to the antagonistic action on ER-dependent signaling. Zhang et al. demonstrated that both TCDD and the relatively non-toxic AHR agonist 6-methyl-1,3,8-trichlorodibenzofuran (MCDF) induced CYP1A1-dependent ethoxyresorofin-O-deethylase (EROD) activity and inhibited proliferation of seven ER-negative cell lines (BT474, HCC38, MDA-MB453, MDA-MB-435, MDA-MB-436, MDA-MB-157 and MDA-MB-468) (Zhang, et al., 2009). In the case for MDA-MB-231 cells, Hall et al. demonstrated decreased cell motility, invasiveness, and mammosphere formation in the presence of TCDD and DIM (Hall, et al., 2010). Moreover, Prud'homme et al. clearly demonstrated that tranilast is an AHR agonist with inhibitory effects on BCSCs and was especially effective against CSCs in TNBC specifically selected for drug resistance (Prud'homme, et al., 2010). Therefore, AHR activation presents a strong clinical application for the treatment for breast cancer, especially for the TNBC subtype. However, there are a few studies that contribute AHR activation to increased tumor progression and increased malignancy in ER-negative breast cancer and are discussed below.

One of those studies was conducted by Miret et al., which demonstrated that the progression of TNBC occurs through AHR activation with the carcinogenic environmental molecule hexachlorobenzene (HCB), and resulted in enhanced invasion and migration through the promotion of the Smad, Jun amino-terminal kinase (JNK), and p38 pathway (Mireta, et al., 2016). Enhanced invasion and migration indicates that HCB modulates crosstalk between AHR and TGFβ and consequently exacerbates a pro-migratory phenotype in MDA-MB-231 cells, which contributes to a high degree of malignancy. Another case for AHR activation promoting malignancy in MDA-MB-231 cells was demonstrated by D'Amato et al., as they showed that the tryptophan metabolite kynurenine increased activation of AHR while cells were in suspension, which in turn inhibits the cell death process anoikis during the metastatic process (D'Amato, et al., 2015). Moreover, inhibition or knockdown of tryptophan 2,3-dioxygenase (TDO2) decreased kynurenine production, increased anoikis sensitivity, while inhibiting proliferation, migration, and invasion. Additionally, AHR inhibition or knockdown also decreased proliferation, migration, and anchorage-independent growth. D'Amato et al. revealed that the TDO2-AHR signaling axis activated in TNBC cells promoted anoikis resistance and metastasis, and that pharmacological inhibition of TDO2 decreased lung colonization in a preclinical model of TNBC.

These studies identify AHR as a tumor promoting factor in TNBC. However, there are some therapeutic strategies to consider when incorporating AHR agonists in breast cancer treatment regimens. Even though an endogenous AHR agonist such as kynurenine was able to promote the metastatic process, we are unaware as to how these treatments regulate JAG1 expression. Moreover, kynurenine has been identified to be a weak AHR agonist (Mezrich, et al., 2010), signifying that it has a low binding affinity to the AHR and can possibly be out-competed

with non-toxic AHR ligands that are known to inhibit cancer as well as have a higher binding affinity to the AHR, such as tranilast and ITE (Henry, Bemis, Henry, Kende, & Gasiewicz, 2006). This study allows us to postulate that perhaps these tumor-promoting AHR ligands may not reduce JAG1 expression and may even increase its expression, which only adds to the complexity of the endogenous regulatory roles of AHR and warrants further investigation. However, as stated in the previous section, the role of the AHR in facilitating tumor progression is only manifested in either the absence of a non-toxic exogenous AHR ligand (i.e. tranilast, omeprazole, DIM, IC3) or through the knockdown and inhibition of the AHR (Ikuta, et al., 2000; Al-Dhfyan, Alhoshani, & Korashy, 2017). Considering the fact that AHR signaling plays a critical role in immunity, development, and hematopoiesis, targeting the AHR with antagonists may promote unwarranted side effects during treatment.

Our studies reveal for the first time that TCDD and the endogenous AHR agonist ITE plays a tumor suppressive role in two TNBC cell lines (MDA-MB-231 and MDA-MB-157) (Figures 23, 25-30). We were able to determine that TCDD significantly decreased JAG1 expression after 24 hours in MDA-MB-231 cells, and that ITE was able to not only significantly decrease JAG1 expression after 24 hours but was able to maintain JAG1 suppression over the course of 5 days (Figure 25). Additionally, MDA-MB-231 cells treated with ITE for 3 days were also able to inhibit the Notch signaling pathway because of the decrease in JAG1 (Figure 26). This decrease of JAG1 expression was further indicted by observing decreases in the NICD levels as well as the decrease of direct Notch target gene, HES1. Our AHR knockdown studies were also able to demonstrate that inhibition of the Notch signaling pathway was AHR-dependent, as removal of the AHR prevented ITE from suppressing JAG1 expression and promoted the recovery of the Notch signaling pathway (Figure 27). In fact, in AHR knockdown

cells not treated with ITE, there was significant increase in both JAG1 and NICD levels, indicating that the AHR may act as a tonic JAG1 suppressor (Figure 27). MDA-MB-157 cells were also treated with ITE for 3 days and mirrored the outcome of MDA-MB-231 cells, as the decrease in JAG1 expression resulted in the inhibition of the Notch signaling pathway (Figure 26). From the functional perspective, we were able to demonstrate that the decrease in JAG1 expression from both ITE and JAG1-targeting siRNA inhibited both migration (scratch assay) and invasion (Boyden chamber) of MDA-MB-231 cells (Figure 30).

Collectively, these results along with previously published data identify that activation of the AHR inhibits invasion and migration, primarily through decreasing the expression of JAG1 and ultimately inhibiting the Notch signaling pathway in TNBC cells. Other than regulating migration and invasion, JAG1-dependent Notch signaling also plays an important role in drug-resistance and CSC maintenance (Li, Masiero, Banham, & Harris, 2014). This allows us to hypothesize that ITE may promote inhibitory effects on BCSCs by decreasing JAG1 expression and restore sensitivity to treatment regimens as it was demonstrated with the AHR agonist tranilast. Tranilast was able to enhance treatment response and cytotoxicity in ER-negative breast cancer in conjunction with tamoxifen and induced apoptosis in a synergistic manner (Darakhshan & Ghanbar, 2013).

7.1.3. Preliminary Data

We expanded upon our first report showing that the decrease in JAG1 via TCDD and ITE were AHR-dependent by studying other AHR ligands that have been identified to be non-toxic as well as promote anti-cancer effects. One of the AHR ligands of interest was tranilast, an anti-allergy medication that has gained attention for its anti-metastatic effects that were shown in both *in vivo* and *in vitro* studies (Rogosnitzky, Danks, & Kardash, 2012). We applied tranilast

(200μM) to MDA-MB-231 cells every 24 hours for 1, 3 and 5 days and discovered that tranilast reduced JAG1 on day 3 and day 5 significantly, compared with vehicle-treated controls (Figure 29). Overall, we were able to show that small molecules which are structurally unrelated to each other may be able to promote AHR-dependent anti-cancer effects with the same mechanism, by decreasing JAG1 expression. From this data and our current knowledge, we can now pose a new question: Our laboratory has identified three AHR ligands that induce anti-cancer effects, do other non-toxic AHR ligands reduce JAG1 expression as a common mechanism behind their anti-cancer effects?

Even though TCDD has been identified as a carcinogen, its ability to inhibit cancer progression *in vitro* as well as in breast cancer patients who've been exposed to this chemical after diagnosis has also been documented (Murray, Patterson, & Perdew, 2014). This presented a unique paradox, where AHR activation was identified to possess both pro-cancer and anti-cancer affects and developed a schism between researchers in terms of identifying the endogenous function of the AHR as well as the strategy for targeting AHR signaling in cancer therapies. Based on the findings from our research, we hypothesized that decreases in JAG1 would decrease the expression of JAG1-dependent downstream target genes that have previously been identified to promote cancer progression, such as Snail (Chen, Imanaka, Chen, & Griffin, 2010) (Figures 33 and 34). Interestingly, we discovered that AHR knockdown breast cancer cells via AHR-targeting siRNA are unable to suppress JAG1 levels in the presence of both TCDD and ITE (Figure 27). In fact, MDA-MB-231 cells treated with AHR-targeting siRNA showed that JAG1 expression was significantly increased in the untreated AHR-targeting siRNA treated group compared to the untreated control siRNA group (Figure 27). Our findings in this study complemented those found by Safe et al., leading to the discovery of a novel pathway by which

the AHR functions to modulate the Notch signaling pathway as a method for suppressing cell cycle progression and metastasis in response to various non-toxic small molecules from both endogenous (produced via metabolism by the micro flora in the GI tract) and exogenous (present due to diet, environment, and medication regimen) sources (Safe, Cheng, & Jin, 2017). This research regarding the diet and microbiome composition could help propose another potential therapy target for treating breast cancer as well as provide new insight on how small molecules from our environment and diet can potentially regulate developmental signaling pathways and the induction of corresponding changes in cellular physiology. With this information we can pose two questions: 1) Does AHR activation also change the expression levels of JAG1 target genes that are known to promote cancer progression? 2) How do AHR ligands that have been identified as tumor-promoting, besides TCDD, effect JAG1 expression as well as target genes downstream of JAG1-dependent signaling?

As promising and novel as this discovery is, what is critical to note is that even though the decrease in JAG1 expression is AHR-dependent, it is not a direct AHR target gene (GEO accession number GSE98515). Our research led to a discovery that uncovers an extremely important connection between the small molecules present in our environment and cancer progression through the modulation of developmental signaling pathways. However, the mechanism behind AHR-dependent JAG1 suppression has yet to be completely understood. A viable mechanism can potentially be determined by identifying if any of the 189 TCDD-treated AHR-direct target genes (Table 7) can also act as a repressor of JAG1 or if AHR decreases expression of a certain gene that is known to promote JAG1 expression. Elucidating the complete mechanism can serve a critical importance in determining the differences between the endogenous functionality of the AHR in the both cancerous and non-cancerous cell lines,

considering that increased JAG1 protein levels in cancer cells correlate with increased aggressiveness and metastatic potential while normal epithelial type or non-malignant cells have little to no JAG1 expression (Cohen, et al., 2010). Therefore, it is important to ask the questions: 1) What are some AHR-direct target genes that can act as a suppressor of JAG1 expression? 2) Does AHR inhibit expression of a gene that promotes JAG1 expression?

We studied the regulation of JAG1 via ITE in two ER-positive cell lines (MCF7 and T47D) and two TBNC cell lines (MDA-MB-231 and MDA-MB-157) (Figures 19-29). Surprisingly, when we treated HCC1134 TNBC cells with ITE every 12 hours for 5 days, JAG1 expression was increased significantly compared to the control group (Figure 35), which is completely opposite of the results in MDA-MB-231 cells. However, the levels of the AHR in HCC1143 cells were significantly lower compared to MDA-MB-231 cells (Figure 35). These results tell us that the amount of AHR present within the cell can potentially determine the direction JAG1 is regulated as well as may be used as a biomarker to determine the efficacy of treatment with AHR agonists. This further suggests that suppressing expression of JAG1 is an endogenous function of AHR, where low AHR levels resulted in the promotion of JAG1 expression in the presence of ITE. Moreover, this observation may also shed insight on how AHR signaling can promote both pro-cancer and anti-cancer effects via activation of the AHR as well as how the initial amount of JAG1 and or AHR in cancer cells may potentially give rise to different cellular outcomes. Additionally, the increase in JAG1 levels may also occur because HCC1143 cells fall under a different sub-category of TNBC cells compared to MDA-MB-231 cells, as HCC1143 are classified as basal-like, while MDA-MB-231 are classified as mesenchymal-like. This observation can be justified based on an extensive study conducted by Yamamoto et al. that demonstrated NF-κB-dependent induction of JAG1 and the Notch-

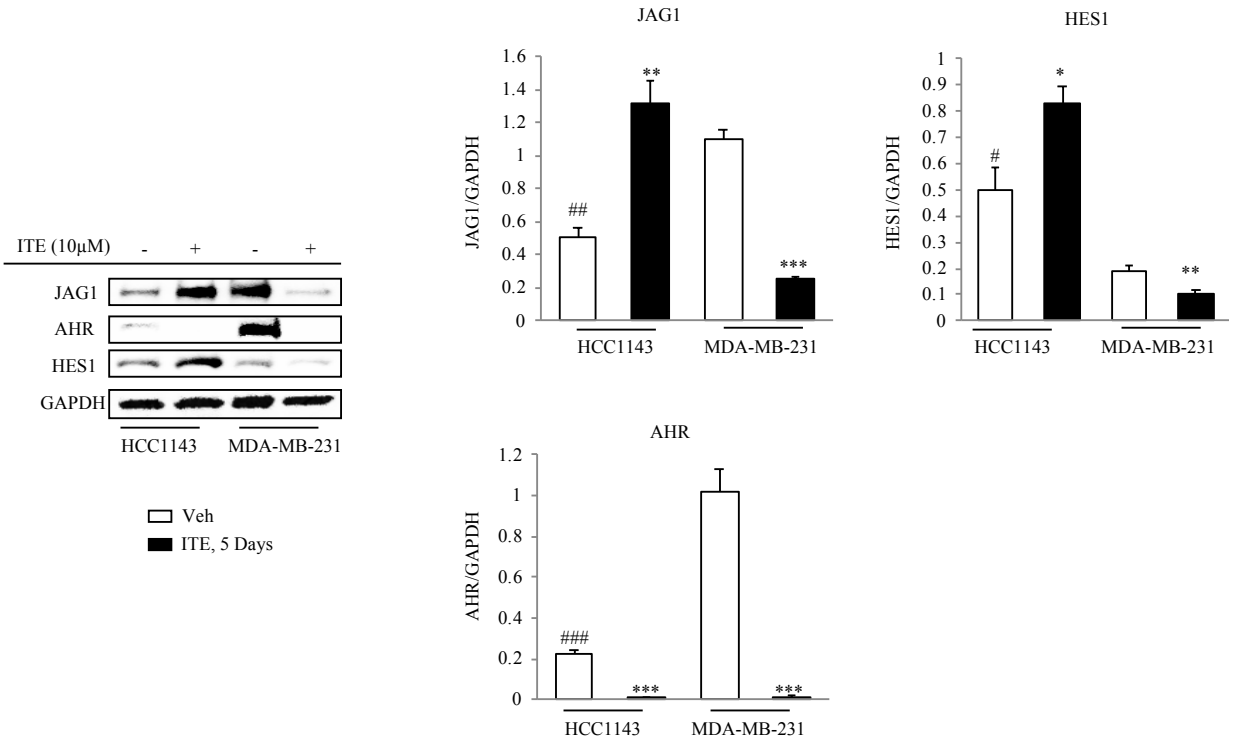


Figure 35. Activation of AHR via ITE Promotes JAG1 Expression in HCC1143 Breast Cancer Cells

HCC1143 and MDA-MB-231 cells were treated with vehicle or 200μM Tranilast every 24 hours for 5 days. Total cellular protein was isolated and subjected to western blot analysis. Levels of JAG1 and HES1 were normalized to GAPDH. Results shown are the means ± SEM of three independent experiments. Significant differences in untreated HCC1143 cells compared with untreated MDA-MB-231 cells are indicated by $^{\#}P < 0.05$, $^{\#\#}P<0.01$, or $^{\#\#\#}P<0.001$. Significant changes in expression by ITE are indicated by *P < 0.05, **P<0.01, or ***P<0.001.

dependent expansion of the CSC population occurs only in the basal-like subtype of TNBC (Yamamoto, et al., 2013). Ultimately, this observation can lead us to ask the question: Could direct JAG1 inhibition via monoclonal antibodies or small molecule inhibitors be a novel chemotherapy tactic for the treatment of breast cancer as it may by-pass the controversy behind the use of AHR agonists in the treatment for breast cancer?

In short, we have uncovered several novel roles for the AHR in breast cancer. The AHR is required for the anti-cancer effects of non-toxic AHR ligands in breast cancer cells (Hall, et al., 2010). TCDD, a prominent and well-studied AHR ligand, exposed the mechanism behind the anti-cancer effects of AHR (Murray, Patterson, & Perdew, 2014). These anti-cancer effects resulted in the discovery of non-toxic AHR ligands that are currently produced endogenously by the diet and certain medications (Hubbard, Murray, & Perdew, 2015). Finally, ITE (Cheng, et al., 2015) and tranilast (Rogosnitzky, Danks, & Kardash, 2012) treatment studies revealed that JAG1 (Li, Masiero, Banham, & Harris, 2014) is required for the proliferation, invasion and cell-movement of TNBC cells and that various targets downstream of JAG1-dependent signaling are also regulated by activation of the AHR. The next section will discuss the preliminary data that we currently have that can help answer some of the questions that were mentioned in this section, as well as provide insight for future projects surrounding the relationship between JAG1 and the AHR.

7.2. Rationale for New Experiments

We hypothesize that other non-toxic AHR ligands induce their anti-cancer effects by decreasing the expression of JAG1 and thereby reducing the expression levels of other oncogenes that require JAG1-dependent Notch signaling. The rationale for this hypothesis stems from prior reports showing that JAG1 is increased in more aggressive cancer types and is known

as an indicator of poor prognosis (Cohen, et al., 2010). Upon activating the Notch signaling pathway, JAG1 induces gene expression that promotes various functions in cancer biology including but not limited to metastasis, EMT, proliferation, drug-resistance, and CSC maintenance (Li, Masiero, Banham, & Harris, 2014). The activation of the AHR is highly relevant to the suppression of breast cancer, considering that overexpression of JAG1 is sufficient to induce growth of normal mammary cells and induce mesenchymal characteristics (Duryagina, et al., 2013). The JAG1 gene is not only amplified in more aggressive subtypes of breast cancer but also in ovarian cancer (Steg, et al., 2011), brain cancer (Purow, et al., 2005), and various types of leukemia (Li, Masiero, Banham, & Harris, 2014). In this regard, one study noted JAG1 was critical for MDA-MB-231 breast cancer growth *in vitro* and *in vivo* (Chen, et al., 2016). Our preliminary data demonstrate that the amount of AHR protein present within the cell may perhaps determine the regulatory outcome of JAG1 expression, as we showed that ITE-treated HCC1143 TNBC cells increased JAG1 expression and that the AHR levels are much lower compared to the MDA-MB-231 cell line (Figure 35). Given the previous reports and current data, we hypothesize that regulation of JAG1-dependent signaling via activation of the AHR could be determined by the amount of AHR protein that is present within the cell.

High JAG1 expression is linked to promoting metastasis in various cancer types and Snail expression has also been shown to be a necessity for the metastatic process. Snail is required for promoting the transformation of epithelial cells into mesenchymal cells, in order to induce cell-movement and invasion (Chen, Imanaka, Chen, & Griffin, 2010). This is because Snail inhibits the expression of E-cadherin by binding directly to its promoter and blocking its transcription, thereby suppressing the characterizations of the epithelial cell type. Our preliminary data demonstrate that AHR signaling decreases the expression of JAG1 and Snail via

TCDD and ITE (Figures 20-22, 24-26, 33) and we provide the first evidence that this could be mediated in an AHR-dependent manner (Figures, 23, 27-28, 34). Given the previous reports and our current data, we hypothesize that the decrease in JAG1 expression reduces the levels of downstream target genes of JAG1-dependent Notch signaling such as Snail as the mechanism for the anti-metastatic activity of AHR activation.

It is well established that the Notch pathway and, in particular, JAG1-dependent Notch activation plays an important role in tumor biology because it affects both cancer cells and multiple components of the vascular and immunological microenvironment. It has also been shown that excess Notch activation transforms normal breast cells found in pre-invasive and invasive human breast cancer and correlates with early recurrence (Li, Masiero, Banham, & Harris, 2014). Moreover, inhibition of Notch signaling reduced BCSC activity (Simões, et al., 2015). However, the major concern for targeting the Notch pathway by GSIs was the GI toxicity that resulted in severe diarrhea; as the Notch signaling pathway is necessary to maintain the goblet cell population to maintain consistent water absorption in the GI tract (Katoh & Katoh, 2007). Furthermore, GSIs may target proteases other than γ-secretase, which could result in unwarranted regulatory array of cellular functions. In order to overcome such limitations, recent studies have shown natural products that are non-toxic to humans, such as DIM (Hall, et al., 2010), IC3 (Weng, Tsai, Kulp, & Chen, 2008), and other indoles and isoflavones (Sarkar, Li, Wang, & Kong, 2010) could potentially inhibit the metastatic and invasion process via reducing the expression of Notch receptors and/or Notch receptor ligands. Interestingly, many of these natural products act as AHR ligands. Given this known data, we hypothesize that the endogenous AHR ligand ITE can increase sensitivity to current cancer therapy agents such as tamoxifen. The following sections will detail future experiments that will be done to test our hypothesis.

7.3. Characterizing the Role of AHR-dependent Regulation of JAG1 in Other Breast Cancer Cell Lines Using ITE

An agonist of the AHR was isolated from porcine lung tissue and identified as ITE. Three experiments support the conclusion that ITE is an AHR agonist: 1) ITE was shown to compete with radioactive TCDD for binding to the AHR in human, murine, killifish, and zebrafish (Song, et al., 2002), 2) Saturation-binding isotherms indicated a high affinity interaction between ITE and the AHR (Henry, Bemis, Henry, Kende, & Gasiewicz, 2006) and 3) ITE treatments changed the AHR to its DRE-binding conformation and promoted CYP1A1 expression in a concentration- and time-dependent manner (Henry, Bemis, Henry, Kende, & Gasiewicz, 2006; Song, et al., 2002). Furthermore, administration of ITE to pregnant mice led to AHR signaling in fetal tissues without the toxicities related to TCDD exposure, indicating *in vivo* bioactivity (Nugent, et al., 2013).

Given our current data and knowledge, AHR signaling promotes a suppressive effect on JAG1 expression potentially by the given amount of AHR protein present in the cell (Figure 35). These experiments would establish if the AHR levels, which vary between cell lines, is responsible for the regulatory outcome of JAG1 expression. Two TNBC cell lines showed differential regulation in JAG1 expression, which can potentially be linked to the amount of AHR protein present in the cell (Figure 35). This is because the levels of the AHR in HCC1143 cells were significantly lower compared to MDA-MB-231 cells (Figure 35). This data also mimicked the results from the AHR knockdown experiments conducted in MDA-MB-231 cells when treated with ITE, where loss of the AHR removed the ability for ITE to reduce JAG1 expression (Figures 23 and 27). Therefore, it is imperative to conduct ITE treatments in various breast cancer cell lines, as well as non-cancerous mammary cell lines, to help identify and

establish the true nature of the AHR and its role in the regulation of JAG1 expression.

Non-cancerous MCF-10A cells, ER-positive breast cancer cells (MCF7, T47D, BT474 and SUM185), ER-negative breast cancer cells with the basal-subtype (SUM190, HCC1143, MDA-MB-468), ER-negative with the mesenchymal subtype (BT549, MDA-MB-231 and MDA-MB-157) and the HER2-overexpressing subtype (MDA-MB-453 and SKBR3) would be plated in 6-well plates at a density of 200,000 cells/mL and grown to full confluency before being treated every 12 hours for 3 days with either ITE (10μM) or control vehicle. Cells will be rinsed with PBS, and total cellular extract will be collected in SDS sample buffer [40% glycerol, 8% SDS, 5% BME, 0.04% bromophenol blue in Tris-HCl pH 6.8]. Proteins will be heat denatured and then separated by SDS/PAGE followed by transfer to PVDF membrane. The blots will be incubated overnight at 4 °C while rocking in primary antibody followed by an incubation period of 90 minutes in secondary antibody at room temperature (37°C). Blots will then be rinsed five times (5 minutes per rinse) with PBST before undergoing chemiluminescence to identify JAG1, cleaved-NICD, HES1, and AHR protein concentrations before and after ITE treatment; GAPDH will used as a loading control. ChemiDoc MP Imaging System will be utilized to quantify band density and acquire western blot images.

A two-tailed unpaired t-test will be used to determine significant changes in AHR, JAG1, HES1 and cleaved-NICD, due to the TCDD and ITE activating Notch signaling in ER-positive breast cancer (MCF7 and T47D), while inhibiting it in ER-negative breast cancer (MDA-MB-231 and MDA-MB-157) and that ITE increased JAG1 and HES1 protein expression in HCC1143 breast cancer cells.

Cell lines that experienced significant reductions or increases in JAG1 expression will undergo AHR knockdown with AHR-targeting siRNA, to determine if these changes in JAG1

expression are AHR-dependent. In brief, cell suspensions of 200,000 cells/mL concentrations will be mixed with 100nM of AHR siRNA with 3μL of Lipofectamine RNAiMAX in DMEM/FBS (10%) for ~20 minutes. Cells will then be immediately plated on 35mm tissue culture plates in DMEM/FBS (10%) and cultured for 48 hours before being treated with 10μM ITE every 12 hours for 3 days, followed by western blot analysis of GAPDH (loading control), JAG1, cleaved-NICD, HES1 and AHR. For significance to be determined, a one-way ANOVA will be used along with a Tukey's Post-hoc test, to compare changes in protein levels among the different groups.

To determine if ITE affects cell migration and invasion, scratch and Boyden Chamber assays will be conducted on cell lines that regulated expression of JAG1. For the scratch assay, cells will be plated in 12-well tissue culture plates at a concentration of 50,000 cells/well for 24 hours prior to treatment. The cells will be treated with vehicle or 10μM ITE in DMEM/FBS (1%) every 12 hours for 5 days, then a scratch will be made in each well with a pipette tip. Media will be aspirated to remove floating cells. Vehicle (DMSO) or 10μM ITE will be reapplied in DMEM/FBS (1%). The scratches will be photographed at 0 hours and at 24 hours post scratch using the Lycia Microscope and the exposed surface area of the plates will be measured using ImageJ analysis software. For the Boyden chamber assays, cells will be plated on 35 mm tissue culture plates in DMEM/FBS (10%) for 24 hours prior to treatment. After cells are treated with vehicle or 10μM ITE in DMEM/FBS (10%) every 12 hours for 5 days, they will be detached from tissue culture plates (using trypsin) and counted. 100,000 cells will be immediately transferred to cell invasion chambers in 500μL of media. Chambers filled with cells will be incubated in 24-well tissue culture plates containing DMEM/FBS (10%) as the chemoattractant and incubated for 36 hours. Following the incubation in the presence of vehicle (DMSO) or

10μM ITE, a cotton swab will be used to remove non-adherent cells that were not invasive. Invasive cells will be stained with crystal violet for 10 minutes at room temperature, then rinsed gently with water and then incubated in DMSO for 10 minutes with orbital shaking to extract crystal violet. Cell lysates will be measured at 560nm for invasive activity. For the indicated siRNA experiments, the cell invasion will be assayed 48 hours after transfection.

7.4. Characterizing the Regulation of JAG1 and the Anti-cancer Effects of ITE Using an in vivo Model

To confirm the regulation of JAG1 and its corresponding anti-cancer effects *in vivo*, female NOD *scid* gamma mice (8 weeks of age and weighed 18-22 grams) will be injected unilaterally with MDA-MB-231 cells ($2.5x10^6$ cells/animal) in 200μL of 50:50 Matrigel/Collagen I into the fourth abdominal fat pad by subcutaneous injection at the base of the nipple as described by Iorns et al (Iorns, et al., 2012). When the tumor volume reaches approximately $110mm^3$, the mice will be divided into homogeneous blocks based on their tumor volumes followed by randomly assigning each block into the vehicle control and ITE treatment group (N = 5/group). The vehicle (DMSO) or ITE (80 milligrams (mg)/mL in DMSO) will be administered by intraperitoneal injection once daily for 28 days at a volume of 1mL/kg body weight.

A gross body weight and tumor volume will be determined twice weekly using a scale and calipers expressed in mm^3, respectively. Tumor weights will be converted from tumor volumes by assuming a tumor density of 1 mm^3 = 1 mg. A net body weight will be obtained by subtracting a tumor weight from a gross body weight. After the final injection, mice will be given an additional day to document the tumor volume, body weight, and other clinical signs before mice are euthanized by CO_2 asphyxiation. At the time of sacrifice, a macroscopic

examination of metastases will be noted in lung, liver, and kidney; any visible metastatic foci will be counted.

For western blot analysis, primary tumor tissue samples will be collected and homogenized in lysis buffer containing protease inhibitors. The protein will be collected via centrifugation and concentrations will be determined by a bicinchoninic acid protein assay. 40μg of total protein will be boiled in SDS sample buffer [40% glycerol, 8% SDS, 5% BME, 0.04% bromophenol blue in Tris-HCl pH 6.8] for 10 minutes and then separated by SDS/PAGE followed by transfer to a PVDF membrane. The blots will be incubated overnight at 4°C with rocking in primary antibody followed by an incubation period of 90 minutes in secondary antibody at room temperature (37°C). Blots will then be rinsed five times (5 minutes per rinse) with PBST before undergoing chemiluminescence to identify JAG1, cleaved-NICD, HES1, and AHR protein concentrations before and after ITE treatment; GAPDH will used as a loading control. ChemiDoc MP Imaging System will be utilized to quantify band density and acquire western blot images.

7.5. Determining the Effects of ITE in Combination with Tamoxifen in Breast Cancer Cells

Darakhshan & Ghanbar demonstrated that the combination between tranilast and tamoxifen enhanced anti-tumor effects in both ER-positive (MCF-7) and ER-negative cells (MDA-MB-231) as this particular combination resulted in synergistic effects on growth, proliferation, TGFβ signaling as well as vascular endothelial growth factor (VEGF) and MMP9 expression (Darakhshan & Ghanbar, 2013). These studies suggest that combining non-toxic AHR agonists such as ITE with current breast cancer chemotherapies as a novel approach to increase treatment efficacy for both ER-positive and ER-negative breast cancer. This combination could be particularly effective in patients with aggressive cancer types such as ER-

negative breast cancer due to increased dependency of JAG1-Notch signaling (Li, Masiero, Banham, & Harris, 2014). Regarding ER-positive breast cancer, ~50% of breast cancer patients treated with tamoxifen experienced tumor reoccurrence combined with drug-resistance due to increased JAG1 expression in the CSC population in breast tumors (Simões, et al., 2015). Therefore, utilizing a non-toxic AHR agonist such as ITE may help target the CSC population in drug-resistant breast cancer cells due to its ability to decrease JAG1 expression.

For cell culture and treatments, MCF-7 and MDA-MB-231 breast cancer cells would be grown and maintained at 37 °C with 5% CO_2 in DMEM/FBS (10%). The concentrations of tamoxifen used will be 1, 2, 5, 10, and 20μM, concentrations of ITE will be 0.01, .1, 1, 10, 100μM, and the combination treatment will be conducted in 2μM tamoxifen with a range of concentrations of ITE (0.01, 0.1, 1, 10, 100μM) every 12 hours for 3 days.

For western blot analysis, after 3 days of treatment, cells will be rinsed with PBS and total cellular extract will be isolated in 250μL of SDS sample buffer [40% glycerol, 8% sodium SDS, 5% BME, 0.04% bromophenol blue in Tris-HCl pH 6.8]. Proteins will be heat denatured and then separated by SDS/PAGE followed by transfer to PVDF membrane. The blots will be incubated overnight at 4°C with rocking in primary antibody followed by an incubation period of 90 minutes in secondary antibody at room temperature (37°C). Blots will then be rinsed five times (5 minutes per rinse) with PBST before undergoing chemiluminescence to identify JAG1, cleaved-NICD, HES1, and AHR protein concentrations before and after ITE treatment; GAPDH will be used as a loading control. ChemiDoc MP Imaging System will be utilized to quantify band density and acquire western blot images. A two-tailed unpaired t-test will be conducted for each concentration to determine significant changes in protein expression between treated and untreated cells.

For RT-qPCR, total RNA will be extracted using RNA purification columns (Qiagen). Reverse transcription will be performed on 300ng of RNA using cDNA synthesis kits (Applied Biosystems (Foster City, CA)) in accordance with the suppliers' instructions. Real-time PCR will be conducted with the StepOnePlus (Applied Biosystems) using SYBR green master mix (Applied Biosystems) in accordance with the suppliers' protocols. Samples will be analyzed in triplicate and the average will be normalized to GAPDH loading control. Relative changes in gene expression will be quantitated using the $2^{-\Delta\Delta CT}$ formula. A two-tailed unpaired t-test will be conducted for each concentration to determine significant changes in mRNA expression between treated and untreated cells.

One property of BCSCs is the ability to form colonies in soft agar, so a colony forming assay will be conducted (Prud'homme, et al., 2010; Cheng, et al., 2015). Briefly, 20,000 cells in 0.5% agar will be layered on preformed 0.8% agar layer using a 35mm non-tissue culture dish. The cells in agar will be treated with either vehicle, 2μM tamoxifen, 10μM ITE or a combination of both in DMEM/FBS (10%) every 12 hours for 7 days. Colonies will be counted under a microscope using low magnification (4x) and photographed after 7 days.

To identify any changes in cell population, a cell proliferation assay will be conducted. $1x10^4$ cells/well of either MCF-7 or MDA-MB-231 will be transferred to 96-well plates and be incubated overnight. When treatments begin, 300μL of fresh medium with the indicated drug concentrations will be added to each well and incubated. Treatments will be replenished every 12 hours for 3 days and will also be tested in triplicate as well as replicated independently three times. After 72 hours, 20μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5mg/mL in PBS) will be added to each well and incubated for 4 hours. The media with MTT will be discarded and 100μL of DMSO will be added to dissolve formazan

crystals at room temperature for 30 minutes. The optical density of each well will be determined at 570nm and the percentage of cell viability will be calculated according to the following equation: Cell viability (%) = [A_{570}(sample)/A_{570}(control)] x 100.

To determine changes in migration, a scratch assay will be conducted in 12-well tissue culture plates at a concentration of 50,000 cells/well and grown to full confluency prior to treatment. Cells will be treated with vehicle, 2μM tamoxifen, 10μM ITE or a combination of both every 12 hours for 3 days before a scratch is made in each well with a pipette tip. Media will be aspirated to remove floating cells and vehicle treatments will be reapplied in DMEM/FBS (1%). The scratches will be photographed at 0 hour and at 24 hours post scratch using the Lycia Microscope. The exposed surface area of the plates will be measured using ImageJ analysis software.

To determine changes in invasion, a Boyden chamber assay will be conducted. Cells will be plated on 35 mm tissue culture plates in DMEM/FBS (10%) for 24 hours prior to treatment. After cells are treated with vehicle, 2μM tamoxifen, 10μM ITE or a combination of both every 12 hours for 3 days, the cells will be detached from tissue culture plates (using trypsin) and counted. 100,000 cells will be immediately transferred to cell invasion chambers in 500μL of media. Chambers filled with cells will then be incubated in 24-well tissue culture plates containing DMEM/FBS (10%) as the chemoattractant for 36 hours. Each experiment will include a negative control in which chambers filled with cells will be incubated with DMEM lacking FBS. Following the 36 hour incubation period in the presence of vehicle (DMSO) or 10μM ITE, a cotton swab will be used to remove non-adherent cells that were not invasive. Invasive cells will be stained with crystal violet for 10 minutes at room temperature and then rinsed gently with deionized water. Stained cells will be incubated in DMSO for 10 minutes with orbital shaking to

extract crystal violet. Cell lysates will be measured at 560nm for invasive activity.

7.6. Characterizing JAG1 Regulation with Other Non-toxic AHR Ligands

Given our current data and knowledge, AHR promotes a suppressive effect on JAG1 expression in breast cancer cells when treated with ITE (Figures 24-29). Currently, there are known AHR agonists that have been documented to inhibit cancer growth and progression; however, the mechanisms behind their anti-cancer effects have yet to be determined (Weng, Tsai, Kulp, & Chen, 2008; Jin, 2011). A promising and productive approach for developing anti-cancer agents has been the repositioning of pharmaceuticals that were originally developed for other purposes (Safe, Cheng, & Jin, 2017). Some AHR-active pharmaceuticals such as tranilast and omeprazole may be effective AHR-dependent anti-cancer agents for single or combination cancer chemotherapies for treatment of breast cancers. Other diet based AHR agonists such as IC3 and DIM have also been identified as potential drug candidates (Hall, et al., 2010; Weng, Tsai, Kulp, & Chen, 2008). Therefore, it is imperative to conduct experiments in breast cancer cell lines that responded to ITE in order to help determine if the regulation of JAG1 expression possesses a common mechanistic outcome among other non-toxic AHR ligand treatments. Table 12 depicts a summary of the known effects of non-toxic AHR in various breast cancer cell lines along with the changes in functional outcomes.

7.6.1. JAG1 and Tranilast

Tranilast is an orally active non-toxic drug that is used for the treatment of allergies and can be taken daily (Rogosnitzky, Danks, & Kardash, 2012). There is clear evidence for the anti-cancer effects of tranilast in both *in vivo* and *in vitro* models, through the inhibition of both cell proliferation and cell cycle progression in several mammary carcinoma cell lines, including the human lines MDA-MB-231, MCF-7, and BT- 474 (Chakrabarti, Subramaniam, Abdalla, Jothy,

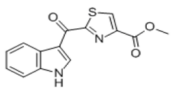
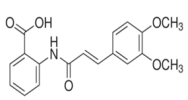
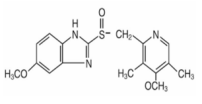
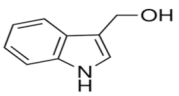
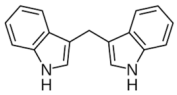
	ITE	Tranilast	Omeprazole	IC3	DIM
Molecular Structure					
Tested Breast Cancer Cell Lines	MCF7 T47D MDA-MB-231 MDA-MB-157	MCF7 MDA-MB-231 BCSCs BT-474	MCF7 MDA-MB-231	MFC7 MDA-MB-231 MDA-MB-468	MCF7 T47D MDA-MB-231 SKBR3
Targeted Signaling Pathways	TGF-β Notch	TGF-β MAPK	N/A	Estrogen pAkt NF-κB	
Targeted Oncogenes **(Decreased Expression)**	JAG1 Snail Oct4	Bcl-2 Oct4 SNAIL Cluster of Differentiation 135	MMP9 CXCR4	Cyclin E Cyclin D1 Bcl-2 VEGF MMP9 Cyclin-dependent Kinase 6	COX2 Survivin SOX4 Bcl-2 Cyclin-dependent Kinase 6
Functional Outcome	Reduced migration and invasion	Reduced mammosphere formation, proliferation and invasion	Reduced migration and invasion	Induced cell cycle arrest and decreased proliferation, migration, and invasion	Reduced invasion and migration

Table 12. Currently Known Anti-cancer Effects Contributed by Non-toxic AHR Ligands

& Prud'homme, 2009; Prud'homme, et al., 2010; Darakhshan & Ghanbar, 2013). Moreover, tranilast was then identified as an AHR agonist with inhibitory effects against CSCs from TNBC selected for anti-cancer drug resistance by inhibiting colony formation, mammosphere formation and stem cell marker expression. Knowing that JAG1 promotes the BCSC phenotype, it is important to determine if tranilast reduces JAG1 expression in breast cancer cells and to identify if the regulation is AHR–dependent.

Non-cancerous MCF-10A cells, ER-positive breast cancer cells (MCF7, T47D, BT474 and SUM185), ER-negative breast cancer cells with the basal-subtype (SUM190, HCC1143, MDA-MB-468), ER-negative with the mesenchymal-subtype (BT549, MDA-MB-231 and MDA-MB-157) and the HER2-overexpressing subtype (MDA-MB-453 and SKBR3) will be treated, processed, and analyzed in the same manner described in section 7.3., but instead of ITE, breast cancer cells lines will be treated with either 200μM tranilast or vehicle (DMSO).

7.6.2. JAG1 and Omeprazole

Omeprazole is a well-known and commonly used proton pump inhibitor that has been identified to clearly inhibit MDA-MB-231 cell migration and invasion in *in vitro* models (Jin, Lee, Pfent, & Safe, 2014). This response was attenuated after knockdown of the AHR by siRNA or after co-treatment with AHR antagonists. Moreover, these *in vitro* assays were complemented by inhibition of lung metastasis of MDA-MB-231 cells in mice that had cells administered via tail vein injection and treated with 200μM omeprazole and resulted in decreased expression of pro-metastatic genes MMP9 and CXCR4 in a AHR-independent and –dependent manner, respectively (Jin, Lee, Pfent, & Safe, 2014). Interestingly, Notch signaling increases expression of CXCR4 in order to maintain the CSC phenotype in renal cell carcinoma and promotes migration in ovarian cancer (Chillakuri, et al., 2013). Therefore, it is imperative to conduct

omeprazole treatments in breast cancer cell lines, as well as in non-cancerous mammary cell lines to help identify if the decrease in JAG1 expression acts as the mechanism responsible for inducing anti-cancer effects.

Non-cancerous MCF-10A cells, ER-positive breast cancer cells (MCF7, T47D, BT474 and SUM185), ER-negative breast cancer cells with the basal-subtype (SUM190, HCC1143, MDA-MB-468), ER-negative with the mesenchymal-subtype (BT549, MDA-MB-231 and MDA-MB-157) and the HER2-overexpressing subtype (MDA-MB-453 and SKBR3) will be treated, processed, and analyzed in the same manner described in section 7.3., but instead of ITE, breast cancer cells lines will be treated with either 200μM omeprazole or control vehicle.

7.6.3. JAG1, Indole-3-Carbinol (I3C) and 3,3'-Diindolylmethane (DIM)

Phytochemicals derived from precursor glucosinolates in cruciferous vegetables (i.e. broccoli, cabbage, and cauliflower) such as the indoles have already been used therapeutically and have been shown to possess potent anti-cancer activity (Murray, Patterson, & Perdew, 2014). More importantly, phytochemicals have been the focus of clinical trials due to their reduced side effects in normal cells and pronounced anti-cancer activities (Weng, Tsai, Kulp, & Chen, 2008). Most of the attention has been given to IC3 and its stable condensation product DIM. IC3 was identified to promote anti-cancer activity by increasing expression of microRNA-34a, a tumor suppressive microRNA that is transcriptionally regulated by p53 (Hargraves, He, & Firestone, 2016). Interestingly, *in vitro* studies have determined that miRNA-34a decreases chemotherapy resistance, cell proliferation and metastasis in human breast cancer cells through inhibition of the Notch signaling pathway (Wang, Li, Kong, Ahmad, Banerjee, & Sarkar, 2010b). This is because microRNA-34a targets both Notch1 and JAG1 mRNA and suppresses its translation to protein (Hashimi, et al., 2009). More recently, DIM was also shown to increase the expression of

miRNA-21 and the microRNA-212/132 cluster, which acted as a metastatic suppressor by targeting JAG1 and SOX4, respectively (Hanieh, 2015). Therefore, it is imperative to conduct IC3 and DIM treatments in breast cancer cell lines, as well as in non-cancerous mammary cell lines in order to identify if there is a decrease in JAG1 expression.

Non-cancerous MCF-10A cells, ER-positive breast cancer cells (MCF7, T47D, BT474 and SUM185), ER-negative breast cancer cells with the basal-subtype (SUM190, HCC1143, MDA-MB-468), ER-negative with the mesenchymal-subtype (BT549, MDA-MB-231 and MDA-MB-157) and the HER2-overexpressing subtype (MDA-MB-453 and SKBR3) will be treated, processed and analyzed in the same manner described in section 7.3., but instead of ITE, breast cancer cell lines will be treated with either 200μM I3C, DIM 25μM DIM, or control vehicle.

7.7. Identifying Potential AHR-dependent Mechanisms that Regulate JAG1 Expression in Breast Cancer Cells

Even though the decrease in JAG1 expression is AHR-dependent, it is important to note that JAG1 is not a direct AHR target gene. As a result, the mechanism behind AHR-dependent JAG1 suppression has yet to be completely understood. There are logical hypotheses that can explain this outcome in various ways. It is important to note that the RNA-seq data from the TCDD treated MCF7 breast cancer cells were analyzed 6 hours after a 100nM TCDD treatment, signifying that the reduction of JAG1 mRNA as a rapid and early occurrence that may occur without binding directly to the JAG1 promoter. Therefore, one of the mechanisms can involve activation of a gene that suppresses JAG1 expression, which can potentially be identified from any of the 41 TCDD/AHR-direct target genes that can also act as a repressor of JAG (Table 9). This data set can also identify a second potential mechanism, which involves AHR activation

increasing expression of certain microRNAs that are known to target JAG1 mRNA and lead to the decrease of JAG1 protein levels. Lastly, another potential AHR-dependent mechanism that decreases JAG1 levels can occur via crosstalk with other signaling pathways by squelching co-factors away from the NF-κB signaling pathway proteins. Each of these proposed potential mechanisms will be reviewed in detail along with experimental designs in the following sections.

7.7.1. JAG1 and HES1

One potential mechanism that can potentially explain how the AHR decreases JAG1 expression is by increasing the expression of a gene that suppresses JAG1 expression, which can potentially be one of the 189 TCDD-treated AHR direct target genes (Table 7). Several lines of evidence suggest that activation of the AHR reduces the expression of JAG1 by increasing HES1 expression. Even though HES1 is a direct target gene of the Notch signaling pathway and has been identified as an indicator for Notch signaling activation (Borggrefe & Liefke, 2012), HES1 also acts to suppress JAG1 expression as a negative-feedback mechanism for the Notch pathway (Bray, 2006). Moreover, differential gene expression analysis shows that HES1 mRNA is induced 1.8-fold after a 6 hour 100nM TCDD treatment and TCDD-stimulated AHR ChIP-seq analysis has identified AHR binding sites in the HES1 gene (Table 9). Another prior report has demonstrated that HES1 is a direct AHR gene target in T47D breast cancer cells (Kobayashi, et al., 2009). More importantly, HES1 is a transcriptional repressor that binds to HES1 sites in the JAG1 gene and inhibits the transcription of JAG1 in embryonic stem cells by binding directly to the JAG1 promotor to suppress its transcription. Stable increases in HES1 would explain how AHR activation via ITE induces long-term suppression (5 days) of JAG1 in MDA-MB-231 cells (Figure 25), while the AHR is rapidly degraded by the proteasome soon after AHR activation (Figure 15). Therefore, it is imperative that we conduct AHR knockdown experiments, in order

to study if the increased expression of HES1 plays a role in the regulation of JAG1 in the presence of ITE and to determine if any change in regulation is AHR-dependent.

Non-cancerous MCF-10A cells and ER-positive (MCF7, T47D, BT474 and SUM185) breast cancer cells will undergo AHR knockdown transfections with HES1-targeting siRNA, in order to determine if these reductions in JAG1 expression are AHR-dependent. In brief, cell suspensions at 200,000 cells/mL will be mixed with 100nM of AHR-siRNA with 3μL of Lipofectamine RNAiMAX in DMEM/FBS (10%) for ~20 min. Cells will be immediately plated on 35mm tissue culture plates in DMEM/FBS (10%) and cultured for 48 hours before being treated with ITE (10μM) every 12 hours for 3 days, followed by western blot analysis of GAPDH (loading control), JAG1, cleaved-NICD, HES1 (positive control) and AHR. For significance to be determined for reduction in the levels of JAG1 and HES1 protein, a one-way ANOVA will be used along with a Tukey's Post-hoc test, to compare the changes in protein among the four groups.

To determine if HES1 knockdown affects cell migration and invasion in the presence of ITE, scratch and Boyden Chamber assays will be conducted on cell lines that inhibited expression of JAG1. For the scratch assay, HES1-targeting siRNA transfected breast cancer cells will be plated in 12-well tissue culture plates at a concentration of 50,000 cells/well for 24 hours prior to treatment. The cells will be treated with vehicle or 10μM ITE in DMEM/FBS (1%) every 12 hours for 5 days; a scratch will be made in each well with a pipette tip. Media will be aspirated to remove floating cells, then vehicle (DMSO) or 10μM ITE will be reapplied in DMEM/FBS (1%). The scratches will be photographed at 0 hours and at 24 hours post scratch using a Lycia Microscope and the exposed surface area of the plates will be measured using ImageJ analysis software.

For the Boyden chamber assays, HES1-targeting siRNA transfected cells will be plated on 35mm tissue culture plates in DMEM/FBS (10%) for 24 hours prior to treatment. After cells are treated with vehicle or 10μM ITE in DMEM/FBS (10%) every 12 hours for 5 days, they will be detached from tissue culture plates (using trypsin) and counted. 100,000 cells will be immediately transferred to cell invasion chambers in 500μL of DMEM. Chambers filled with cells will be incubated in 24-well tissue culture plates containing DMEM/FBS (10%) as the chemoattractant for 36 hours. Following an incubation period of 36 hours in the presence of vehicle (DMSO) or 10μM ITE, a cotton swab will be used to remove non-adherent cells that were not invasive. Invasive cells will be stained with crystal violet for 10 minutes and then rinsed gently with tap water and then incubated in DMSO for 10 minutes with orbital shaking to extract crystal violet. Cell lysates will be measured at 560nm for invasive activity.

7.7.2. JAG1 and microRNA-21

MicroRNAs are attractive candidates as upstream regulators of metastatic progression because they can post-transcriptionally regulate entire sets of genes. There are a few examples of AHR ligands that regulate microRNAs and in turn promote anti-tumor effects. DIM was identified to induce microRNA-146a expression and inhibited cancer cell invasion by suppressing EGFR, NF-κB and metastasis-associated protein 2 (MTA2) (Li, et al., 2010). DIM also upregulated let-7b and miR-200c in gemcitabine-resistant pancreatic cancer cells, causing a reversal of EMT via suppression of zinc finger E-box binding homeobox 1 (ZEB1), Slug, and Vimentin, which in turn induced up-regulation of E-cadherin (Li, et al., 2009). TCDD and DIM were also able to suppress breast cancer metastasis by increasing the expression of the microRNA-212/132 cluster, which targeted SOX4 expression (Hanieh, 2015). Collectively,

identifying microRNAs that are regulated by the AHR but also target JAG1 mRNA are viable candidates for ITE- and TCDD-mediated JAG1 suppression.

Therefore, another possible mechanism for the decrease in JAG1 expression is that the AHR may also indirectly promote the expression of miR-21, a microRNA that targets JAG1 mRNA and prevents its translation into protein (Selcuklu, Donoghue, Kerin, & Spillane, 2012). Selcuklu et al. were able to demonstrate that MCF7 cells stimulated with estrogen showed increased expression of JAG1. In contrast to JAG1, miR-21 is downregulated by estrogen mediated signaling and is negatively correlated in breast cancer cells. When estrogen is applied to MCF7 cells, miR-21 expression is down-regulated through a regulatory site in the miRNA-21 producing gene (MIRN21) promoter region. The ability for the AHR to potentially promote expression of miR-21 was already demonstrated in a study showing that the AHR ligand DIM increased miR-21 expression and targeted cell division cycle 25A (cdc25A) in MCF7 cells, resulting in decreased cell proliferation (Jin, 2011). However, increases in miR-21 expression have been demonstrated with other AHR ligands. Therefore, TCDD and ITE may promote the expression of miR-21 by inhibiting its estrogen-mediated suppression and may potentially serve as the mechanism behind decreased JAG1 expression in ER-positive breast cancer cells.

Non-cancerous MCF-10A cells and ER-positive (MCF7, T47D, BT474 and SUM185) breast cancer cells will be cultured in DMEM/FBS (10%) at 37°C, then plated in 6-well plates at a density of 200,000 cells/mL and grown to full confluency. To induce JAG1 expression, cells will be treated with estrogen (50nM) overnight before being treated every 12 hours for 3 days with either ITE (10μM) or control vehicle. Cells will be rinsed with PBS, and total cellular extract will be isolated in SDS sample buffer [40% glycerol, 8% SDS, 5% BME, 0.04% bromophenol blue in Tris-HCl pH 6.8]. Proteins will be heat denatured and then separated by

SDS/PAGE followed by transfer to PVDF membrane. GAPDH loading control western blot will be used to confirm equal protein loading (~15μL/sample). The blots will be incubated overnight at 4°C with rocking in primary antibody followed by an incubation period of 90 minutes in secondary antibody at room temperature. Blots will then be rinsed five times (5 minutes per rinse) with PBST before undergoing chemiluminescence to identify JAG1 and AHR protein concentrations before and after ITE treatment. ChemiDoc MP Imaging System will be utilized to quantify band density and acquire western blot images. A one-tailed unpaired t-test will be conducted to determine significant changes in protein expression between treated and untreated cells.

In order to determine if AHR regulates miR-21 expression and if miR-21 regulates JAG1 expression, transient transfections with siRNA will be performed as described in Section 4.4. In brief, cell suspensions (200,000 cells/1mL) will be mixed with 100nM of either miR-21-targeting siRNA, AHR-targeting siRNA or control-siRNA with 3µL of Lipofectamine RNAiMAX in DMEM/FBS (10%) for ~20 minutes before being treated every 12 hours for 3 days with either ITE (10µM) or control vehicle.

RT-qPCR will be used to measure changes in JAG1 mRNA and miR-21 expression. Total RNA will be extracted using RNA purification columns (Qiagen). Reverse transcription will be performed on 300ng of RNA using cDNA synthesis kits (Applied Biosystems (Foster City, CA)) in accordance with the suppliers' instructions. StepOnePlus (Applied Biosystems) will be used to take PCR measurements. Amplifications will be carried out using SYBR green master mixes (Applied Biosystems) according to the manufactures protocols. Samples will be done in triplicate and the average normalized to GAPDH will be quantified using the $2^{-\Delta\Delta CT}$ formula.

In order to determine if the JAG1 3'-untranslated region (UTR) is targeted by miR-21 in ER-positive breast cancer cells, reporter constructs and a luciferase assay will be conducted. The JAG1 3'-UTR region (632 bp) harboring the miR-21 binding site will be PCR amplified from human genomic DNA, cloned into endonuclease Spel/Hind III restriction sites in the pMIR-REPORTS luciferase vector, designated as pMIR/JAG1 3'-UTR. A mutated sequence will be generated using Quikchange Lightning SDM kit, designated as pMIR/JAG1-UTRdel. Cells will be co-transfected with 300ng of each plasmid (pMIR/JAG1-UTR, pMIR/JAG1-UTRdel, or pMIR) and 1ng of phRL renilla vector (for normalization) in 24-well plates. After 24 hours of transfection, a dual luciferase assay will be performed according to the manufacturer's protocol.

7.7.3. JAG1 and NF-κB Signaling

Even though increases in HES1 and miR-21 are prime candidates for the suppression of JAG1 expression in the presence of TCDD and ITE, these mechanisms may not apply to the ER-negative breast cancer subtype because HES1 expression was suppressed in MDA-MB-231 and MDA-MB-157 cells (Figures 26). Moreover, miR-21 expression was regulated in an ER-dependent manner and was shown to be unaltered in MDA-MB-231 cells in the presence of estrogen according to the study conducted by Selcuklu et al. (Selcuklu, Donoghue, Kerin, & Spillane, 2012). It is therefore unlikely that TCDD or ITE reduces JAG1 expression in MDA-MB-231 and MDA-MB-157 through increased expression of miR-21 and may occur through a different mechanism.

It is possible that the decrease in JAG1 expression in ER-negative breast cancer cells can potentially occur though a crosstalk mechanism that involves AHR activation and the NF-κB signaling pathway (Figures 13 and 32). TNBC exhibits high levels of constitutively active NF-κB signaling by inflammatory cytokines and induces JAG1 expression in order to promote

Notch-dependent expansion of the CSC population (Yamamoto, et al., 2013). Yamamoto et al. were able to clearly demonstrate that JAG1 expression is NF-κB-dependent in TNBC. Moreover, this study was also able to determine that the NF-κB signaling pathway is highly activated in basal-like (HCC1143) and mesenchymal-like (MDA-MB-231) subtype tumors and is significantly positively-correlated with JAG1 expression. Interestingly, the expression levels of other Notch ligands (i.e. JAG2 and DLL4) were not significantly affected by NF-κB signaling, indicating that JAG1 is a unique gene that is specifically regulated by NF-κB in TNBC.

Specifically, in MDA-MB-231 cells, activation of the NF-κB signaling pathway via TNFα did not induce expression of JAG1 mRNA or protein, although the induction of RelB, a known NF-κB-inducible gene, was observed. On the contrary, JAG1 expression was significantly decreased upon exposure to TNFα after 24 hours and signifies that JAG1 expression is not induced by the non-canonical NF-κB pathway via RelB in MDA-MB-231 cells (Figure 13). Rather, JAG1 expression is most likely regulated by the canonical NF-κB pathway through RelA in MDA-MB-231 cells, which was demonstrated by Bash et al. in HeLa-derived HtTA-1 cells (Bash, et al., 1999) (Figures 13 and 31). However, it is important to note that TNFα induces JAG1 expression via RelB in the basal-like (HCC1143) subtype. Therefore, JAG1 expression is likely regulated by the non-canonical pathway in addition to the canonical pathway, signifying that the level of total NF-κB activation (the sum of canonical and non-canonical activation) may determine the level of JAG1 expression (Yamamoto, et al., 2013). The ability for the activation of the AHR to cross-talk with the NF-κB signaling pathway has been well-documented and was reviewed in detail in the third chapter (Vogel, et al., 2007; Tian, 2009; Sheppard, et al., 1998) (Figure 13). In short, activation of the AHR promoted direct protein binding between the AHR and RelA, proposing that the mutual repressive effects were

modulated by the formation of inactive AHR-RelA dimers, which in turn reduces the concentration of available nuclear AHR protein and RelA needed for AHR-NF-κB-mediated gene expression (Tian, 2009). This cross-talk interaction can potentially reduce expression of JAG1 in MDA-MB-231 and MDA-MB-157 cells and therefore may promote the ability for the AHR to induce anti-metastatic effects in the mesenchymal subtype of TNBC.

Non-cancerous MCF-10A cells, ER-negative breast cancer cells with the basal-subtype (SUM190, HCC1143, MDA-MB-468), ER-negative with the mesenchymal-subtype (BT549, MDA-MB-231 and MDA-MB-157) and the HER2-overexpressing subtype (MDA-MB-453 and SKBR3) will be cultured in DMEM/FBS (10%) at 37°C, then plated in 6-well plates at a density of 200,000 cells/mL and grown to full confluency before being treated every 12 hours for 3 days with either ITE (10μM) or control vehicle. For western blot analysis, after 3 days of treatment cells will be rinsed with PBS, and total cellular extract will be isolated in 250μL of SDS sample buffer [40% glycerol, 8% SDS, 5% BME, 0.04% bromophenol blue in Tris-HCl pH 6.8]. Proteins will be heat denatured and then separated by SDS/PAGE followed by transfer to PVDF membrane. The blots will be incubated overnight at 4 °C with rocking in primary antibody followed by an incubation period of 90 minutes in secondary antibody at room temperature. Blots will then be rinsed five times (5 minutes per rinse) with PBST before undergoing chemiluminescence to identify JAG1, cleaved-NICD, RelA, RelB, and AHR protein concentrations before and after ITE treatment; GAPDH will used as a loading control. ChemiDoc MP Imaging System will be utilized to quantify band density and acquire western blot images. A two-tailed unpaired t-test will be conducted for each cell-line in order to determine significant changes in protein expression between treated and untreated cells.

In order to determine if RelA and RelB regulates JAG1 expression, transient transfection with siRNA will be performed as described in Section 4.4. In brief, cell suspensions (200,000 cells/mL) will be mixed with 100nM of either RelA-targeting siRNA, RelB-targeting siRNA, or control-siRNA with 3µL of Lipofectamine RNAiMAX in DMEM/FBS (10%) for ~20 minutes before being treated every 12 hours for 3 days with either ITE (10µM) or control vehicle.

In order to determine if heterodimers RelA-AHR or RelB-AHR are formed in the presence of ITE, a co-immunoprecipitation (Co-IP) experiment will be conducted. After the 3 day ITE treatment, cells will be washed twice with cold PBS, harvested by scraping, and collected by centrifugation at 1500 x g. Cells will then be lysed in lysis buffer (20 millimolar (mM) 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES), pH 7.4, 125mM sodium chloride (NaCl), 1% Triton X-100, 10mM ethylenediaminetetraacetic acid (EDTA), 2mM ethyleneglycol-bis-(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 2mM sodium orthovanadate (NA_3VO_4), 50mM sodium fluoride (NaF), 20mM zinc chloride ($ZnCl_2$), 10mM sodium pyrophosphate ($Na_4P_2O_7$), 1 mM phenylmethylsulfonyl fluoride (PMSF), and 5µg/mL leupeptin) and centrifuged for 15 minutes at 12,000 x g, and supernatant fraction will be collected. Anti-AHR antibodies will be added to the lysate, and the binding reactions will be performed at 4°C for 2 hours on a rotary shaker, then 30µL of GammaBind Plus Sepharose beads will be added to precipitate the antibody-antigen complex. The beads will be washed 3 times in lysis buffer and boiled in 2x SDS sample buffer and undergo western blot analysis with anti-RelA and anti-RelB primary antibodies at a 1:1000 concentration and corresponding secondary antibody.

To determine if ITE affects RelA-AHR and RelB-AHR heterodimers binding to the JAG1 promoter, a ChIP assay will be conducted. Cells (500,000 per 60mm plate) will be plated

and grown in DMEM/FBS (10%) before being treated with ITE or DMSO for 3 hours. Formaldehyde (1%) will be added to the medium for 10 minutes, followed by glycine (.5 molar (M)) for 5 minutes. Cells will then be rinsed with PBS, collected in PBS, pelleted by centrifugation, and lysed in 300μL of lysis buffer (1% SDS, 5mM EDTA, 50mM Tris-HCl, pH 8) per 60mm plate plus protease inhibitors for 15 minutes on ice. Cell extracts will be sonicated (5 times, 10 seconds each) and diluted 1:10 in dilution buffer (16.7mM Tris-HCl, pH 8, 167mM NaCl, 1.2 mM EDTA, 0.01% SDS, 1.1% Triton X-100), rotated overnight at 4°C with 1μg of non-specific IgG, Anti-RelA, or Anti-RelB antibody. Antibody-chromatin complexes will be collected using 5 μL of magnetic protein A beads with rotation at 4°C for 90 minutes. Using magnetic separation, beads will be washed sequentially with buffer 1 (20mM Tris-HCl, pH 8, 150 mM NaCl, 2 mM EDTA, 0.1% SDS), buffer 2 (20mM Tris-HCl, pH 8, 500 mM NaCl, 2 mM EDTA, 0.1% SDS), buffer 3 (10mM Tris-HCl (pH 8), 0.25 lithium chloride (LiCl), 1 mM EDTA, 1% NP-40, 1% deoxycholate), and then 1X 10mM Tris-HCl with 0.1mM EDTA (TE) buffer for 5 minutes each and incubated at 65°C for 4 hours in elution buffer (1% SDS, 0.1M sodium bicarbonate ($NaHCO_3$)) with proteinase K. DNA will be purified and analyzed using RT-qPCR. Primers spanning RelA and RelB response elements in the JAG1 promoter and DREs in the CYP1A1 promoter will be used.

7.8. Identifying Snail as a JAG1 Downstream Target Gene that Promotes Invasion and Migration

It has been proposed that the initial steps in metastasis involve an EMT-like process, which is the process that converts cells from an epithelial, non-motile morphology to become migratory and prone to invade other tissues (Hanahan & Weinberg, 2000). EMT is accompanied by specific changes in gene expression, such as down-regulation of E-cadherin, by which its

promoter is repressed by several transcriptional repressors, including the zinc finger transcription factor Snail (Wang, et al., 2009). Up-regulation of Snail correlates with metastasis and poor prognosis, whereas silencing of Snail is critical for reducing tumor growth and invasiveness (Wang, et al., 2009). Knowing that JAG1-dependent Notch signaling promotes expression of various target genes that are required to promote cancer growth at various stages of tumor progression, we questioned if whether the downstream target genes are also affected by ITE and TCDD treatments in breast cancer cells. Furthermore, Notch can up-regulate Snail and induce EMT in a normoxic environment during normal development in cardiac cell differentiation (Timmerman, et al., 2004), although the detailed molecular mechanism for how Notch controls Snail expression remains to be established. Sahlgren et al. were able to further demonstrate that Notch signaling controls Snail expression by two distinct but synergistic mechanisms, involving both direct transcriptional activation of Snail and an indirect mechanism operating via LOX, leading to elevated Snail protein levels in cervical, colon, glioma, and ovarian cancer (Sahlgren, Gustafsson, Jin, Poellinger, & Lendahl, 2008).

We found a significant reduction in the levels of Snail protein in both ER-positive and ER-negative breast cancer cells treated with ITE and TCDD compared with control cells treated with vehicle (Figures 33 and 34). Our preliminary data demonstrates that AHR decreases the expression of JAG1 and Snail, and we provide the first evidence that this could be mediated through an AHR-dependent manner (Figure 34). Given the previous reports and our current data, we hypothesize that the decrease in JAG1 expression reduces the levels of downstream target genes of JAG1-dependent Notch signaling such as Snail as the mechanism for the anti-metastatic activity of AHR activation. We observed that cells with AHR knockdown were not responsive to ITE-stimulated reductions in Snail, indicating that the suppressive effect of ITE on Snail

expression is transmitted through the AHR (Figure 34). However, it has not been determined if Snail expression is regulated in a JAG1-dependent manner in breast cancer cells.

Non-cancerous MCF-10A cells, ER-positive breast cancer cells (MCF7, T47D, BT474 and SUM185), ER-negative breast cancer cells with the basal-subtype (SUM190, HCC1143, MDA-MB-468), ER-negative with the mesenchymal-subtype (BT549, MDA-MB-231 and MDA-MB-157) will be treated, processed, and analyzed in the same manner described in section 7.4.

In order to determine if JAG1 regulates Snail expression in the presence of ITE, transient transfection with short interfering RNA (siRNA) will be performed as described in section 4.4. In brief, cell suspensions (200,000 cells/mL) will be mixed with 100nM of JAG1-siRNA or control-siRNA with 3µL of Lipofectamine RNAiMAX in DMEM/FBS (10%) for ~20 minutes before being treated every 12 hours for 3 days with either ITE (10µM) or control vehicle.

7.9. Anticipated and Alternative Outcomes for Proposed Experiments

Given our preliminary data showing TCDD and ITE can stimulate a marked decrease in JAG1 expression in ER-positive (MCF7 and T47D) and ER-negative (MDA-MB-231 and MDA-MB-157) breast cancer cells, I expect that cell lines with high levels of AHR and JAG1 expression will inhibit the expression of JAG1. This decrease in JAG1 expression in turn will reduce the migratory and invasive activity of breast cancer cells that rely on JAG1-dependent signaling. Moreover, I expect to see the basal-like subtypes (SUM190, HCC1143, MDA-MB-468) to either increase JAG1 expression or have no significant change when treated with ITE compared to the control group.

Given our current data and previous literature showing that the combination of non-toxic AHR agonists with current anti-estrogenic treatments proves to be an effective treatment for ER-

positive breast cancer, I expect cell colony formation, proliferation, invasion, and metastasis to be inhibited in a synergistic manner in the ITE and tamoxifen combination treatment (Darakhshan & Ghanbar, 2013). Additionally, I expect the anti-proliferative effects to be more prominent in MCF7 cells compared to that of MDA-MB-231 cells due to ITE not affecting cell proliferation in ER-negative breast cancer cells (Figure 32). I also expect to see decreases in JAG1 expression in both cell lines and for the Notch pathway to be inhibited in only MDA-MB-231 cells. As for the MCF7 cells, I suspect the Notch signaling pathway to be affected in the presence of both ITE and tamoxifen, as estrogen signaling promotes Notch-mediated tumor growth and proliferation (Selcuklu, Donoghue, Kerin, & Spillane, 2012). Moreover, I suspect that combinations that include an anti-estrogen treatment such as tamoxifen and a Notch inhibitor may be effective in ER-positive breast cancer (Rizzo, et al., 2008).

Regarding other non-toxic, anti-cancer AHR ligands, I expect that tranilast will inhibit the expression of JAG1, which in turn will also reduce the migratory and invasive activity of breast cancer cells. I also believe the basal-subtype cell lines may exhibit decreases in JAG1 levels, due to tranilast also being identified as a TGFβ inhibitor, a known promoter of JAG1 expression (Rogosnitzky, Danks, & Kardash, 2012). I expect that omeprazole will inhibit the expression of JAG1 in an AHR-dependent manner, which in turn also reduces the migratory and invasive activity of breast cancer cells. However, cell lines with low AHR levels such as HCC1143 may exhibit increases or no change in JAG1 levels, which may not affect the overall physiology of those particular cell lines. Given the data we produced and published literature, I also expect that I3C and DIM will inhibit the expression of JAG1 in an AHR-dependent manner, which in turn may also reduce the migratory and invasive activity of breast cancer cells. Similar to omeprazole, I expect basal-like ER-negative cell lines such as HCC1143 to exhibit increases or no change in

JAG1 levels, which may not affect the overall physiology of those particular cell lines.

In regard to the potential AHR-dependent mechanisms that suppress JAG1 expression in ER-positive breast cancer, I expect that cell lines transfected with HES1-targeting siRNA will inhibit the ability of ITE to decrease expression of JAG1 in an AHR-dependent manner, which in turn would not affect the migratory and invasive activity of breast cancer cells. I also expect ITE to increase miR-21 expression in the presence of ITE compared to the control group in ER-positive breast cancer cells and that knockdown of AHR prevents increased expression of miR-21, which prevents the decrease in JAG1 expression to occur in the presence of ITE. Moreover, I also expect to see that miR-21 knockdown cells prevent the suppression of JAG1 mRNA in the presence of ITE. Finally, I predict that the JAG1 3'- UTR is targeted by miR-21 in ER-positive breast cancer cells. It is also possible that these two mechanisms could occur simultaneously and or in synchronicity, as they operate via independent pathways. However, more research needs to be conducted to investigate this co-operative inhibition.

As for the regulation of JAG1 in the ER-negative basal-like breast cancer subtype, I expect to see ITE induce AHR-RelB heterodimer formation and promote the expression of JAG1 by binding to the JAG1 promoter, that RelB knockdown prevents increases in JAG1 expression, and that RelA knockdown does not alter changes in JAG1 expression. In the mesenchymal-subtype, I expect to see ITE induce AHR-RelA heterodimer formation and inhibit the expression of JAG1 by decreasing the amount of RelA binding to the JAG1 promoter, that RelA knockdown prevents the suppression of JAG1, and that RelB knockdown does not alter changes in JAG1 expression.

In regard to identifying potential downstream targets of JAG1-dependent signaling that promotes the metastatic process, I expect to see the decrease in Snail expression in ITE-treated

ER-positive breast cancer cells as well as mesenchymal-subtype ER-negative breast cancer cells compared to the untreated group and that it occurs in a JAG1-dependent manner. In the basal-like subtype of ER-negative breast cancer, there may be either no change or a promotion in Snail expression due to the unaffected or increased expression in JAG1 upon ITE exposure.

7.10. Conclusion

Investigating the changes in JAG1 levels in the presence of both known anti- and pro-cancer AHR ligands can provide important insight on how the expression of JAG1 in cancer cells as well as changes in cell migration and invasion are altered in a ligand-dependent manner (Hall, et al., 2010). These anti-cancer effects and changes in cell migration and invasion were confirmed by treating MDA-MB-231 breast cancer cells with both TCDD and ITE in the absence of AHR (siRNA) and an AHR antagonist, CH-223191 (Figures 23, 27-28). CH-223191 is reported to selectively antagonize the binding of halogenated AHR ligands to the AHR binding site and was able to prevent TCDD-stimulated reductions in JAG1 expression, but not ITE-stimulated reductions. Not only does this finding indicate that ITE decreases JAG1 expression through a different binding site used by TCDD, ITE binds AHR even in the presence of CH-223191. This data allows researchers to come to an understanding that the basis of information which supports both the use of AHR agonists and antagonists in the treatment of various cancers is initially valid and logical. Because TCDD is a known carcinogen, the use of AHR antagonists such as CH-223191 prevented TCDD from inducing toxicity, originally giving the AHR the role as a tumor promoter. However, as researchers began to see the anti-cancer effects from non-halogenated AHR ligands produced from either the natural products present in the diet or medications being taken, a paradox started to take form and further increased the debate on the role of the AHR in cancer biology.

Therefore, considering all the evidence being presented in this dissertation promoting the use of non-toxic and clinically relevant AHR agonists for the treatment of breast cancer, I can safely oppose the use of AHR antagonists and inhibitors for the treatment of breast cancer. The opposition to utilize AHR antagonists is due to two main factors, which include: 1) research claiming that AHR promotes toxicity and cancer progression utilized highly stable known carcinogenic compounds that possess no clinical relevance or weak endogenous AHR agonists that could be out-competed by exogenous non-toxic AHR agonists with higher AHR-binding affinity and 2) inhibition of AHR through the use of antagonists or inhibitors to treat breast cancer can potentially cause unwarranted long-term side-effects in patients undergoing treatment due to the endogenous roles the AHR regulates which include, but are not limited to immunity, development, and hematopoiesis. Therefore, the use of AHR antagonists and inhibitors for the purpose of cancer treatment could result in the inability to fight infections, reduce wound healing efficiency, and induce anemia.

Moreover, the case where ITE increases JAG1 expression in the basal-like ER-negative subtype (HCC1143) also allows us to propose that establishing a treatment strategy by targeting JAG1 directly through the use of monoclonal antibodies, small molecule inhibitors, or JAG1 mRNA suppression as a method for the treatment of breast cancer. This proposal is due to a number of factors, including: 1) research showing that JAG1-dependent Notch signaling promotes various bio-functions that contribute to cancer progression and does not possess any discrepancy in terms of JAG1 functioning as a tumor suppressor, unlike AHR. Even though there are some cases where Notch signaling acts as a tumor suppressor, none of those studies were linked through activation with JAG1. 2) Targeting JAG1 on the extracellular membrane directly via monoclonal antibodies or small molecules that target JAG1 mRNA and/or its expression to

avoid the use of GSIs, resulting in targeting the Notch pathway that promotes cancer progression and not Notch pathway that is needed for normal cellular homeostasis. 3) Most importantly, this approach may help bypass the controversy for the utilization of AHR agonists for the treatment of breast cancer.

In conclusion, I succeeded in showing that activation of AHR through the use of a non-toxic AHR agonist (ITE) induced inhibition of JAG1 expression in both ER-positive and ER-negative breast cancer cells, which resulted in decreased cell migration and invasion. This research could help propose another potential therapy target for treating breast cancer as well as provide new insight on how small molecules from our environment and diet can potentially regulate developmental signaling pathways and the induction of those corresponding changes in the outcomes of cellular physiology.

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APPENDIX A: OFFICE OF RESEARCH INTEGRITY APPROVAL LETTER

MARSHALL UNIVERSITY
www.marshall.edu

Office of Research Integrity

November 13, 2017

Sean A. Piwarski
Department of Biomedical Sciences
Environmental Toxicology and Cancer Biology Research Cluster
Byrd Biotech Science Building
Marshall University School of Medicine

Dear Mr. Piwarski:

This letter is in response to the submitted dissertation abstract entitled *"Exploring the Regulatory Mechanism of the Notch Ligand Receptor JAGGED1 via the Aryl Hydrocarbon Receptor in Breast Cancer."* After assessing the abstract it has been deemed not to be human subject research and therefore exempt from oversight of the Marshall University Institutional Review Board (IRB). The Code of Federal Regulations (45CFR46) has set forth the criteria utilized in making this determination. Since the study does not involve human subjects as defined in DHHS regulation 45 CFR §46.102(f) it is not considered human subject research. If there are any changes to the abstract you provided then you would need to resubmit that information to the Office of Research Integrity for review and determination.

I appreciate your willingness to submit the abstract for determination. Please feel free to contact the Office of Research Integrity if you have any questions regarding future protocols that may require IRB review.

Sincerely,

Bruce F. Day, ThD, CIP
Director

APPENDIX B: ABBREVIATIONS

3-MC….3-methylcholanthrene

4-HT….4-hydroxytamoxifen

5-FU….5-flurouracil

ABC….ATP binding cassette

ABCG2….ATP binding cassette, sub-family G member 2

ADAM….a-disintegrin and metalloproteinase

ADCs….antibody-drug conjugates

ADH1β….alcohol dehydrogenase 1β

ADP….adenosine diphosphate

AHH….aryl hydrocarbon hydroxylase

AHR….aryl hydrocarbon receptor

AHRR….aryl hydrocarbon receptor repressor

AIs….aromatase inhibitors

Akt…serine-threonine protein kinase

ALDH1….aldehyde dehydrogenase1

ALDH1A3….aldehyde dehydrogenase 1, family member A3

ANOVA….analysis of variance

AP-1….activator protein-1

APC….adenomatous polyposis coli

APEX1….apurinic/apyrimidinic endodeoxyribnuclease 1

AR….androgen receptor

ARNT….aryl hydrocarbon receptor nuclear translocator

ATP….adenosine triphosphate

B[α]P….benzo[α]pyrene

Bcl-2….B-cell lymphoma-2

BCRP….breast cancer resistance protein

BCSCs….breast cancer stem cells

bHLH….basic helix-loop-helix

BLIA….basal-like immune activated

BLIS….basal-like immune suppressed

BME….β-mercaptoethanol

BRG1….brahma-related gene 1

c-myc….v-myc avian myelocytomatosis viral oncogene homolog

c-Rel….v-rel avian reticuloendotheliosis viral oncogene homolog

C2….calcium-binding

CAIX….carbonic anhydrase IX

cAMP….cyclic adenosine monophosphate

CAXII….carbonic anhydrase 12

CBP….CREB binding protein

CCND1….cyclin D1

CCND3….cyclin D3

CCNE….cyclin E

CD133….cluster of differentiation 133

CD24….cluster of differentiation 24

CD34….cluster of differentiation 34

CD44….cluster of differentiation 44

CD46….cluster of differentiation 46

cdc25A….cell division cycle 25A

CDK2….cyclin-dependent kinase 2

CDK8….cyclin-dependent kinase 8

CDKN1A….cyclin-dependent kinase inhibitor 1A

ChIP….chromatin-immunoprecipitation

ChIP-seq….chromatin immunoprecipitation-sequencing

CK1α….casein kinase 1α

CMF…. cyclophosphamide, methotrexate, and fluorouracil

CMML….chronic myelomonocytic leukemia

Co-IP….co-immunoprecipitation

CO_2….carbon dioxide

CoRs….co-repressors

COS2….kinesin motor protein costal-2

COX2….cyclooxygenase 2

CRD….cysteine-rich domain

CREB….cAMP response element binding protein

CSCs….cancer stem cells

CSL…. RBP-Jκ/Su(H)/lag-1

CSN2….casein kinase-β

CtBP….c-terminal binding protein

CTLA4….cytotoxic T-lymphocyte-associated protein 4

CUL4B….cullin 4B

CXCL10….C-X-C motif chemokine ligand 10

CXCR4….C-X-C motif chemokine receptor 4

CYP1A1….cytochrome P450, family 1 subfamily A member 1

CYP1B1….cytochrome P450, family 1 subfamily B member 1

CYP450….cytochrome P450

DCs….dendritic cells

DDB1….damaged DNA binding protein 1

Dhh….desert hedgehog

DICD….DLL intracellular domain

DIM….3,3-diindolylmethane

Dlg….discus large homolog

DLL….delta-like ligand

DLL1….delta-like ligand 1

DLL3….delta-like ligand 3

DLL4….delta-like ligand 4

DM1….emtansine

DMBA….dimethylbenz(a)anthracene

DMEM….Dulbecco's Modified Eagle Medium

DMEM/FBS (1%)….DMEM containing 1% FBS

DMEM/FBS (10%)….DMEM containing 10% FBS

DMSO….dimethyl sulfoxide

DNA ….deoxyribonucleic acid

DRE….dioxin response element

DSL…delta-serrate-lag1

DVL….dishevelled

ECD….extracellular domain

EDTA….ethylenediaminetetraacetic acid

EGF….epidermal growth factor

EGFR….epidermal growth factor receptor

EGTA….ethyleneglycol-bis-(β-aminoethyl ether)-N,N,N’,N’-tetraacetic acid

EMT….epithelial-mesenchymal transition

ER….estrogen receptor

ERE….estrogen response element

EROD….ethoxyresorofin-O-deethylase

FBS….fetal bovine serum

FBXW7….F-box and WD repeat domain containing 7

FDA….Food and Drug Administration

FDR….false discovery rate

FGF….fibroblast growth factor

FGFR2….fibroblast growth factor receptor 2

FICZ….6-formylindolo [3,2-b]-carbazole

Foxp3….forkhead box P3

FSH….follicle stimulating hormone

G-CSF….granulocyte colony-stimulating factor

GAPDH….glyceraldehyde-3-phosphate dehydrogenase

GEO….Gene Expression Omnibus

GHR….growth hormone receptor

GI….gastrointestinal

Gli….glioma associated oncogene

Gli-1….glioma associated oncogene-1

Gli-2….glioma associated oncogene-2

Gli-3….glioma associated oncogene-3

Gli-A….activated Gli

Gli-R….restricted Gli

GM-CSF….granulocyte macrophage colony-stimulating factor

GSCs….glioma stem cells

GSH….glutathione

GSIs….γ-secretase inhibitors

GSK-3β….glycogen synthase kinase-3β

GSK2….glycogen synthase kinase 2

GSTs….glutathione-S-tranferases

HCB….hexachlorobenzene

HDAC1….histone deactylase 1

HDAC2….histone deactylase 2

HEPES….2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid

HER1….human epidermal growth factor receptor 1

HER2….human epidermal growth factor receptor 2

HER3….human epidermal growth factor receptor 3

HER4….human epidermal growth factor receptor 4

HES….hairy enhancer of split

HES1….hairy enhancer of split 1

HES5….hairy enhancer of split 5

HEY….HES related family bHLH transcription factor with YRPW motif

HEY1….HES related family bHLH transcription factor with YRPW motif 1

HGF….human growth factor

Hh….hedgehog

HIF-1α….hypoxia inducible factor 1α

HNSCC….head and neck squamous cell carcinoma

HRE….hypoxic response element

HSCs….hematopoietic stem cells

HSP90….heat shock protein 90

I3C….indole-3-carbinol

ICD….intracellular domain

ICZ.…indolo [3,2-b] carbazole

IDeA….Institutional Development Award

iDRE….inhibitory dioxin response element

IFNγ….interferon-γ

IGF-1R….insulin-like growth factor-1 receptor

Ihh….indian hedgehog

IKKα….inhibitor of NF-κB subunit α

IL-1β….interleukin-1β

IL-10….interleukin-10

IL-17….interleukin-17

IL-2….interleukin-2

IL-22….interleukin-22

IL-6….interleukin-6

IL-8….interleukin-8

IL1R1….interleukin 1 receptor, type 1

ILK….integrin-linked kinase

IPA….Ingenuity Pathway Analysis

ITE….2-(1'H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methylester

IκB….inhibitor of NF-κB

IκBK….inhibitor of NF-κB kinase

JAG1….Jagged1

JAG2….Jagged2

JICD….JAG1 intracellular domain

JNK…. Jun amino-terminal kinase

Kb….kilobases

KIF7….kinesin family member 7

KIT….KIT proto-oncogene receptor tyrosine kinase

LAR….luminal/androgen receptor

LCK….lymphocyte-specific protein tyrosine kinase

LEF1….lymphoid enhancing factor 1

Lfng….lunatic fringe

LH….luteinizing hormone

LiCl….lithium chloride

LOX….lysyl oxidase

LPS….lipopolysaccharide

LRP….low-density lipoprotein receptor

LRP5….low-density lipoprotein 5

LRP6….low-density lipoprotein 6

M….molarity

mAbs….monoclonal antibodies

MAML….mastermind-like

MAP3K1….mitogen-activated protein kinase kinase kinase 1

MAPK….mitogen-activated protein kinase

MCDF….6-methyl-1,3,8-trichlorodibenzofuran

MES….mesenchymal

MIRN21….miRNA-21 producing gene

miRNA-21….microRNA-21

mg….milligrams

mL….milliliter

mm….millimeter

mM….millimolar

MMP2….matrix metallopeptidase 2

MMP7….matrix metallopeptidase 7

MMP9….matrix metallopeptidase 9

mRNA….messenger RNA

MRP1….multidrug resistance-associated protein 1

MT….metallothionein

MTA2….metastasis-associated protein 2

mTOR….mechanistic target of rapamycin

MTT….3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

myc….MYC proto-oncogene

NA_3VO_4….sodium orthovanadate

$Na_4P_2O_7$….sodium pyrophosphate

NaCl….sodium chloride

NAD+….nicotinamide adenine dinucleotide

NaF….sodium flouride

$NaHCO_3$….sodium bicarbonate

NCOA-1….nuclear receptor co-activator-1

NF-κB….nuclear factor-κB

NICD….Notch intracellular domain

NICD1….Notch1 intracellular domain

NICD2….Notch2 intracellular domain

NIK….NF-κB inducing kinase

NLS….nuclear localization sequence

ng….nanograms

nm….nanometer

nM….nanomolar

O_2….oxygen

OCT4….octamer-binding protein 4

OH….hydroxyl group

OX40L….tumor necrosis factor receptor, superfamily member 4

P-gp….P-glycoprotein

p….phosphate group

PAGE….polyacrylamide gel electrophoresis

PAHs….polycyclic aromatic hydrocarbons

PARP….poly-(ADP-ribose) polymerase

PBS….phosphate buffer saline

PBST….PBS with 0.05% Tween-20

pCR….pathologic complete response

PDGF….platelet-derived growth factor

PDGF-β….platelet-derived growth factor-β

PDGFR-β….platelet-derived growth factor receptor-β

PDZ….PSD-95/Dlg/ZO-1

PHAHs….polycyclic halogenated aromatic hydrocarbons

PHD….prolyl hydroxylase domain

PI3K….phosphoinositide-3-kinase

PIK3CA….phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit α

Pin1….prolyl-cis/trans-isomerase 1

PKA….protein kinase A

PMSF….phenylmethylsulfonyl flouride

PPARγ….peroxisome profilerator-activated receptor γ

PR….progesterone receptor

PSD-95….postsynaptic density protein-95

PTCH1….patched1

PTEN….phosphatase and tensin homolog

PVDF….polyvinylidene difluoride

pVHL….von Hippel-Lindau tumor suppressor gene product

qPCR….quantitative polymerase chain reaction

RA….rheumatoid arthritis

RAS….rat sarcoma viral proto-oncogene

RBP-Jκ….recombination signal binding protein for immunoglobulin κJ region

RBX1….ring box1

RelA….v-Rel avian reticuloendotheliosis viral oncogene homolog A

RelB….v-Rel avian reticuloendotheliosis viral oncogene homolog B

RET….ret proto-oncogene

RIN….RNA integrity number

RIP140….receptor interacting protein 140

RNA….ribonucleic acid

RNA-seq….RNA-sequencing

ROS….reactive oxygen species

RT-qPCR….real-time quantitative PCR

RUNX2….runt related transcription factor 2

RUNX3….runt related transcription factor 3

SDS….sodium dodecyl sulfate

SEM….standard error margin

SERDs….selective estrogen receptor down regulators

SERMs….selective estrogen receptor modulators

Shh….sonic hedgehog

SIN3A….histone deactylase complex subunit Sin3a

siRNA….short-interfering RNA

SMO….smoothened

SNAI….snail family of transcriptional repressor

Snail….SNAI1

Slug….SNAI2

Smuc….SNAI3

SOX2….SRY-box 2

SOX4….SRY-box 4

SPEN….sharp and mint

SRY….sex-determining region Y

Su(H)….suppressor of hairless

SUFU….suppressor of fused

T-ALL….T-cell acute lymphoblastic leukemia

T-DM1….Ado-trastuzumab emtansine

TACE….TNFα converting enzyme

TBL3….transducin beta like 3

TCDD….2,3,7,8-tetrachlorodibenzo-p-dioxin

TCF/LEF….T-cell factor/lymphoid enhancing factor

TDO2….tryptophan 2,3-dioxygenase

TE….10mM Tris-HCl with 0.1mM EDTA

TGFα….tumor growth factor α

TGFβ….tumor growth factor β

Th17….T-helper 17

TILs….tumor infiltrating lymphocytes

TKIs….tyrosine kinase inhibitors

TLRs….toll-like receptors

TNBC….triple negative breast cancer

TNF….tumor necrosis factor

TNFα….tumor necrosis factor α

TOP2α….topoisomerase 2α

TP53….tumor protein 53

TP63….tumor protein 63

Treg….T-regulatory

TRGs….TCDD-regulated genes

Tris-HCl….tris-hydrochloride

TSS….transcription start site

TUBB2B….tubulin β, class IIB

Ub….ubiquitin

μg….microgram

μL….microliter

μM….micromolar

μPA….urokinase-type plasminogen activator

UTRs….untranslated regions

VDR….vitamin D receptor

VEGF…. vascular endothelial growth factor

VTCN1….v-set domain containing T-cell activation inhibitor

VWF….von Willebrand Factor

Wnt….wingless-type MMTV integration site family

WV-INBRE….West Virginia-IDeA Networks of Biomedical Research Excellence

XAP2….Hepatitis B virus X-associated protein 2

ZEB1….zinc-finger E-box binding homeobox 1

ZO-1….zona occludens 1

$ZnCl_2$….zinc chloride

αvβ3-integrin….integrin αv and integrin β3

α….alpha

β….beta

γ….gamma

κ….kappa

APPENDIX C: CURRICULUM VITAE

Sean A Piwarski
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Education

July 2012 – September 2017	Doctor of Philosophy in Biomedical Sciences Marshall University, School of Medicine, Huntington, WV
August 2010 – May 2012	Master of Science in Forensic Science Marshall University, School of Medicine, Huntington, WV
August 2006 – May 2010	Bachelor of Science in Biological Sciences Bachelor of Arts in Chemistry California Lutheran University, Thousand Oaks, CA

Research

2012 - Present

Exploring the Regulatory Mechanism of the NOTCH Ligand Receptor Jagged1 via the Aryl Hydrocarbon Receptor in Breast Cancer

- Collaborators: Travis Salisbury, PhD; Estil Hurn, BS; Ateeq Chuadry, BS
- Research conducted at Marshall University School of Medicine as part of the requirement for the degree of Doctor of Philosophy
- Funding: This work was supplemented from Dr. Salisbury through a grant from the WV-INBRE grant and support from the Marshall University Genomics and Bioinformatics Core

Manuscripts Submitted

September 2017

Piwarski, S.A.; Denvir, J; Primerano, D.A.; Fan, J; Salisbury, T.B. Aryl Hydrocarbon Receptor Ligands Reduce JAG1 Expression in Breast Cancer Cells. *Bio. Pharm.* 2017, Under Review

Recent Poster Presentations

March 2017

"The Aryl Hydrocarbon Receptor Decreases Expression of Oncogenic and Embryonic Regulator JAG1 in Triple Negative Breast Cancer"
MUSOM Research Day Marshall University

	School of Medicine, Huntington, WV
April 2016	"Exploring the Mechanism by which TCDD Regulates Jagged1 via the Aryl Hydrocarbon Receptor" CCTS Spring Conference, University of Kentucky Center for Clinical and Translational Sciences, Lexington, KY
March 2016	"Exploring the Mechanism by which TCDD Regulates Jagged1 via the Aryl Hydrocarbon Receptor" MUSOM Research Day Marshall University School of Medicine, Huntington, WV
July 2015	"Exploring the Mechanism by which TCDD Regulates Jagged1 via the Aryl Hydrocarbon Receptor" Appalachian Regional Cell Conference Marshall University, School of Medicine, Huntington, WV

Recent Oral Presentations

February 2017	"Breast Cancer Stem Cells: Potential Chemotherapy Targets and Strategies" State of the Art Review presented to graduate students at Marshall University as part of the 2016 Spring Seminar Series, Huntington, WV
November 2016	"The Extracellular Matrix" Lecture presented for a class to graduate students at Marshall University Biomedical Sciences Program, Huntington, WV

Honors and Awards

October 2014	Chancellor's Scholar Award Recipient • Awarded to underrepresented minorities in the field of science • Funded by the Southern Regional Educational Board 2014-2017
March 2016	1st Place, Graduate Student Poster Presentation, MUSOM Research Day, Marshall University, School of Medicine Huntington, WV
July 2015	2nd Place, Graduate Student Poster Presentation, Appalachian Regional Cell Conference, Marshall University, Huntington WV