

MicroRNA signature in the chemoprevention of breast cancer stem cells by Active  
Hexose Correlated Compound (AHCC)

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# Abstract

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Complementary and Alternative Medicine (CAM) is popularly used among breast cancer patients to improve their quality of life. CAM includes dietary supplements such as Active Hexose Correlated Compound (AHCC®), a cultured mushroom extract shown to positively influence the immune system and cancer. Breast cancer recurrence is believed to be caused by cancer stem cells (CSCs). We postulated that AHCC impacts CSCs epigenetically by targeting miRNA pathways. The effects of AHCC on mammosphere growth were observed in MDA-MB-231, MCF-7, and 4T1 cells. Profiling, RT2-qPCR, and western blot analyses were performed to determine AHCC influence on miRNAs and Tenascin C protein in TNBC cell line MDA-MB-231. Balb/c mice were gavaged with AHCC to examine tumorigenesis effects. Our results demonstrated that AHCC reduced mammosphere growth and cell migration, and upregulated tumor suppressor miR-335 expression in different biological settings. Inhibition of miR-335 increased Tenascin C expression. Consequently, AHCC may influence BCSCs through miRNA pathways.

# Résumé

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Le médecine parallèle et complémentaire (MPC) est populaire chez les patientes atteintes par le cancer de sein. Active Hexose Correlated Compound (AHCC) est un produit MPC, dérivé de la fermentation des champignons, reconnu pour son effet immunoprotecteur et anticancérigène. Les cellules souches cancérigènes (CSCs) sont une sous-population de cellules agressives, résistantes à la chimiothérapie et responsables de récurrence. Notre hypothèse était basée sur le fait que l’AHCC influence de façon épigénétique, et par le biais des microRNAs, la croissance des CSCs. Nous avons observé les effets de l’AHCC sur le développement des mammosphères *in vitro* et *ex vivo* dans le modèle murin Balb/c. Des analyses de profilage, de RT2-qPCR sur les microRNAs, et de transfection des cellules MDA-MB-231 avec miR-335 ont été réalisées. Nos résultats ont démontré que l’AHCC réduit la croissance des mammosphères et la motilité cellulaire, et augmente l’expression miR-335, un suppresseur des tumeurs, ainsi que l’inhibition de Tenascin C dans différents cadres biologiques. Nous concluons que l’effet inhibiteur d’AHCC sur la croissance des cellules souches tumorales est, en partie, dû à la modulation épigénétique par les microRNAs.

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# Abbreviations

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<b>AHCC</b>	Active hexose correlated compound
<b>BALB/c</b>	Bagg albino house mouse
<b>bFGF</b>	Basic fibroblast growth factor
<b>BRCA</b>	Breast cancer gene
<b>CAM</b>	Complementary and alternative medicine
<b>CD24</b>	Glycosylphosphatidylinositol-linked cell surface protein
<b>CD44</b>	Indicative marker for effector-memory T-cells
<b>cDCs</b>	Conventional Dendritic Cells
<b>c-Met</b>	Hepatocyte growth factor receptor
<b>CSC</b>	Cancer stem cell
<b>DMEM-F12</b>	Dubco's modified eagle medium-nutrient mixture F12
<b>E-cad</b>	Epithelial cadherin
<b>EGF</b>	Epidermal growth factor
<b>EMT</b>	Epithelial mesenchymal transition
<b>ER</b>	Estrogen receptor
<b>G1</b>	Cell growth phase 1
<b>GSK-3<math>\beta</math></b>	Glycogen synthase kinase 3 beta
<b>HER2</b>	Human epidermal growth factor receptor 2
<b>HIF</b>	Hypoxia inducible factor
<b>HSC</b>	Hematopoietic stem cell
<b>IFN-<math>\gamma</math></b>	Interferon-gamma
<b>IL</b>	Interleukin
<b>MCF-7</b>	Michigan cancer foundation, ER+ breast carcinoma cell line

<b>MCF-10A</b>	Michigan cancer foundation, breast fibrocystic disease
<b>MDA-MB-231</b>	Epithelial, triple-negative breast cancer cell line
<b>MDR</b>	Multidrug resistance
<b>MET</b>	Mesenchymal-epithelial transition
<b>MSC</b>	Mesenchymal stem cell
<b>Nanog</b>	Transcription factor involved with embryonic stem cells
<b>NK cell</b>	Natural killer cell
<b>Notch</b>	Protein in cell membrane, important in cell signaling
<b>p53</b>	Tumor protein
<b>PR</b>	Progesterone receptor
<b>RPMI</b>	Roswell park memorial institute medium
<b>SLUG</b>	(SNAIL2) Zinc finger protein associated with EMT
<b>SNAIL</b>	(SNAIL1) Zinc finger protein associated with EMT
<b>SOX4</b>	Transcription factor protein encoded by SOX4 gene
<b>STAT</b>	Signal transducer and activator of transcription factor
<b>TGF-<math>\beta</math></b>	Transforming growth factor-beta
<b>TLR</b>	Toll-like receptor
<b>TNBC</b>	Triple negative breast cancer
<b>TNC</b>	Tenascin C
<b>TNF- <math>\alpha</math></b>	Tumor necrosis factor- $\alpha$
<b>TWIST</b>	(TWIST1) basic helix-loop-helix transcription factor protein
<b>Wnt</b>	Wingless-related integration site
<b>ZEB</b>	Zinc finger E-box-binding homeobox
<b>4T1</b>	Metastatic murine cell line

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# **Chapter 1 : Views of CAM use for breast cancer**

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Patient and physician views of CAM use for breast cancer: why it is important to conduct further scientific research on natural products

The chapter begins by briefly mentioning breast cancer statistics and defining CAM. Then we discuss patient and physician views of CAM, lack of knowledge on CAM use, lack of communication between patients and physicians regarding CAM, and current evidence supporting CAM for breast cancer treatment.

Breast cancer is one of the leading causes of cancer-related deaths in Canada, second only to lung cancer, and is the most commonly diagnosed cancer in Canadian women (1). Worldwide, breast cancer affects 1.38 million individuals per year (2). The relative 5-year breast cancer survival rate is as low as 12% in Africa (1997) and as high as 90% in the United States (2007), calculated with total incidence and number of deaths (3). The survival rate has been improving greatly (2), however, there is still much progress to be made (4). Increasingly, breast cancer patients are seeking complementary and alternative therapies to supplement their chemotherapy treatments.

Complementary and alternative medicine (CAM) is the use of medical practices and products that are not presently considered a part of conventional treatment methods within a given country (5). Breast cancer patients use CAM more than patients with other types of cancer, with reports of use as high as 75% in the USA (6,7). Nutritional supplements are popular forms of CAM, including vitamins, herbs and other natural products. This includes the product that we examine in our study, Active Hexose Correlated Compound (AHCC®), used worldwide for immune support, chemoprevention and as an adjunct therapy for many cancer types, including breast cancer. The high CAM use suggests positive patient views of its use for cancer therapy.

Some common reasons breast cancer patients use CAM include: to cure or treat cancer, to prevent cancer recurrence, to reduce chemotherapy side effects, and to boost the immune system (8–12). Breast cancer patients that use CAM tend to be more skeptical of conventional therapies than non-users (8). However, most patients use CAM alongside conventional treatments and report satisfaction with these treatments

(10,11,13,14). Currently, there are several issues arising due to a lack of CAM evidence, including poor patient-physician communication. Since CAM is very commonly used by breast cancer patients, both the patients' and their healthcare providers' perspectives on CAM are important to cancer care, as well as the scientific basis for CAM that influences these perspectives.

Unfortunately, only about 50% of women with breast cancer report their use of CAM to their physician or health care provider (6). This could potentially lead to negative interactions if they are also undergoing a conventional treatment, such as chemotherapy. As an example, antioxidants could potentially diminish the effects of radiation and chemotherapy (15). One study found that less than 5% of women with breast cancer did not disclose CAM use because of fear of their physician's opinion (8). This finding suggests that patients are generally not concerned about physicians giving negative remarks about CAM. Another possibility is that some patients may be worried about insulting their physician, as if mentioning CAM would be suggesting that they are not satisfied with their current care (16). It is also possible that breast cancer patients do not see the necessity of informing their health providers about CAM use. For instance, patients may believe that vitamins are safe to use with medication because they are naturally found in food. It is important to view natural products as medications that could potentially interact with conventional chemotherapy treatments.

An additional issue is that some breast cancer patients will use CAM products without sufficient knowledge on the usage, dosage and potential side effects of these products (15,17). A European study found that only 17.9% of physicians provided CAM

information to patients (18). This may be due to the lack of knowledge of some physicians and due to the lack of evidence-based studies on some CAM products (Table 1.1). This indicates that there is a discrepancy between physician and patient attitudes towards CAM and a lack of communication between the two parties.

**Table 1.1. Physicians' views on cancer patient CAM use.** Positive and negative views held by physicians that influence whether or not they would recommend CAM to their cancer patients.

<b>View</b>	<b>% of physicians</b>	<b>Country</b>	<b>Reporting study</b>
<b>CAM are specifically active against disease</b>	27	Germany	Linde et al., 2015
<b>Insufficient knowledge of CAM to advise patients; want to learn more about CAM</b>	73; 58	New Zealand	Bocock et al., 2011
<b>May be harmful to the patient; want to learn more about CAM</b>	56; 50	USA	Roth et al., 2009
<b>CAM will help patient feel hopeful; would discourage CAM use if disclosed; would cure cancer; would help relieve symptoms</b>	73; 22; 0; 20	Korea	Kim et al., 2008
<b>Discourage CAM use due to lack of knowledge; want to learn more about CAM</b>	61; 81	USA	Milden & Stokols, 2004
<b>Lack of scientific evidence</b>	82	Japan	Hyodo et al., 2003

Despite the skepticism towards CAM by some physicians, there are many studies showing that certain CAM products are beneficial for an array of conditions, including many human clinical trials (19–21) (Table 1.2). The importance is knowing which CAM products are proven effective, and which are needing more research regarding effectiveness and medical interactions.

**Table 1.2. Natural products shown to be effective as CAM for breast cancer patients based on clinical trials.** A selection of popularly used CAM for the prevention and treatment of breast cancer.

<b>Natural Product</b>	<b>Evidence of effectiveness as CAM in human clinical trials</b>
<b>AHCC</b>	Retrospective study demonstrated reduced severity of neutropenia in breast cancer patients (22).
<b>Chinese herbal medicine</b>	Rhodiola alga improved immunity and reduced quantity of oral ulcers caused by chemotherapy in random trial (23).  Traditional Chinese Medicine reduced nausea caused by chemotherapy (24).
<b>Mistletoe</b>	Randomized, double-blind trials with standardized mistletoe extract PS76A2 (Lektinol) improved HRQoL and survival time of breast cancer patients (25,26).
<b>Omega 3 fatty acids</b>	Randomized, double-blind placebo controlled trial found Omega-3s to protect patients against paclitaxel-induced peripheral neuropathy (PIP) which can influence quality of life (27).
<b>Vitamins/minerals</b>	Vitamin C and E administration to breast cancer patients restored antioxidant levels, reduced lipid peroxidation, and reduced DNA damage (28).

Finally, it is important to conduct further research on CAM therapies and products because women with breast cancer may occasionally rely on this form of treatment more than conventional therapies, or may feel that the addition of complementary products to their conventional therapy will provide a better chance to overcome their condition. Research has shown that the perception of cancer patients is an important component of the treatment (29). This effect is even stronger if there is a robust relationship between patient and healthcare provider (29). Consequently, expectations and perspectives of CAM play an important role in breast cancer therapy.

Given that a majority of breast cancer patients use CAM either on its own or alongside conventional treatments, it is important to expand the literature on various forms of CAM. Further research would increase evidence-based knowledge that will benefit both patients' and physicians' attitudes towards CAM.

## Chapter 2 : Background

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The difficulty in treating breast cancer lies in its chemoresistance and high probability of metastasis (4). Diet has been identified as a key priority in achieving dramatic improvements in breast cancer control (30). This current research project is part of efforts to gain a comprehensive understanding of the molecular mechanisms by which natural food components modulate epigenetic changes and cancerous stem cell development in breast carcinoma in in vitro, ex vivo, and in vivo settings.

This chapter will first deal with evidence linking natural products, particularly Active Hexose Correlated Compound (AHCC), to breast cancer treatment. Following, we will discuss how the heterogeneous character of breast cancer makes it a complex disease that requires targeted treatment strategies, with a focus on triple negative breast cancer (TNBC). Furthermore, we will discuss Cancer Stem Cells (CSCs) as new promising targets for breast cancer therapy and important pathways for CSC maintenance and regulation. Additionally, we will discuss mechanisms of CSC resistance to current therapies and a potential protein target involved in this resistance, Tenascin C (TNC). We will explore the specific epigenetic targets within CSCs, specifically novel microRNAs (miRNAs). Finally, we summarize the purpose and objectives of our study.

## 2.1 Natural compounds that target breast cancer: The role of AHCC.

As mentioned in the previous chapter, CAM is very popular among breast cancer patients, including nutritional supplements and natural products. Several natural compounds have been shown to support chemoprevention and therapy in breast cancers including dietary polyphenols from green tea, turmeric, broccoli, and soybeans (31), catechins from green tea (31), folate from spinach, lentils, and almonds (32), vitamin D3 from fish (32). Some of these natural products have also been tested in random controlled trials (33,34). Mushrooms, particularly those rich with  $\alpha$  and  $\beta$  glucans moieties (such as oligosaccharides), are known to have health benefits for cancer as well (35,36).

Among these available natural products is Active Hexose Correlated Compound (AHCC<sup>®</sup>), a cultured mushroom mycelium extract product produced in Sapporo, Japan, mainly recommended for immune support. In Japan, AHCC is the second most popular CAM used by cancer patients (37). Specifically, it is made from the mycelium of *Basidiomycete* mushrooms and consists of amino acids, lipids, minerals, and 74% oligosaccharides, 20% of which are  $\alpha$ -1,4 glucans (38). The acetylated form of these  $\alpha$ -1,4 glucans have a low molecular mass (5kDa) and are believed to be AHCC's active ingredient (38). The beneficial effect of AHCC is believed to result from many compounds in AHCC acting in synergy and targeting global regulators of inflammation that underline many chronic disease including cancer. Although the mechanism of action for AHCC is still unknown, it has been shown to increase the activity of NK cells in people and mice (38,39), prolong the survival and improve the prognosis of patients with

liver cancer (20), help with resistance to bacterial infection in mice (40), improve influenza B resistance in mice (41), enhance CD4+ and CD8+ T cell proliferation in animals and healthy adults (38,42), enhance the antitumor effects of certain chemotherapy drugs in animals (43), and have anti-inflammatory effects in animals (44).

Most AHCC studies have been carried out either in animal models or clinical settings and several doses have been recommended for animals and humans (20,40,43). The recommended daily dose of AHCC for human consumption is 3g/day (45,46). One study reported a 6g/day dose for liver cancer patients (20). A retrospective cohort study of AHCC on breast cancer patients was recently reported. In this study, women were administered 1g of AHCC orally after each meal. After several months, AHCC induced reduction in chemotherapy-induced neutropenia (22). Several animal model studies, looking at the effects of AHCC on a variety of illnesses from influenza to cancer, used doses ranging from 100mg/kg/day to 12.5g/kg/day, with 1g/kg/day being the most frequently used (39,40,43,45,47).

Although many studies have been conducted on AHCC in several cancers, very few studies have examined its effects in breast cancer. Further, its mechanism of action remains to be elucidated. Therefore, in this study we are interested in gaining information on its possible mechanisms of action in breast cancer.

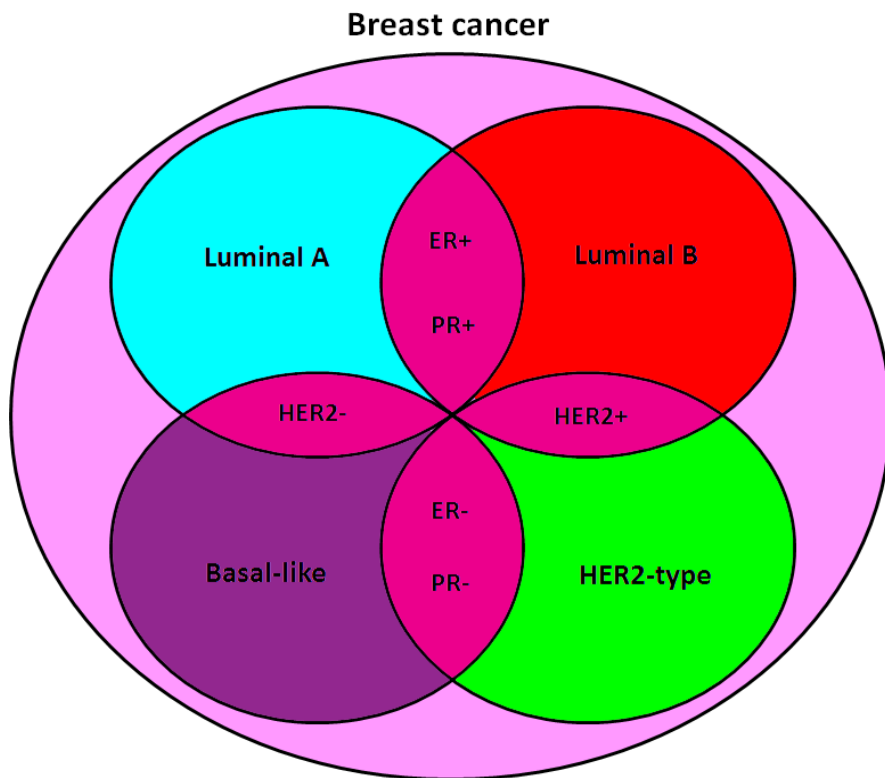
## **2.2 Breast cancer and stem cells**

### **2.2.1 Breast cancer subtypes: An emphasis on triple negative breast cancer (TNBC)**

Breast cancer generally originates in either the breast lobules (glands in which milk is produced) or ducts. Types of breast cancer, based on where the cancer originates, include Ductal Carcinoma In Situ (DCIS), Invasive Ductal Carcinoma (IDC), Invasive Lobular Carcinoma (ILC). DCIS is sometimes called non-invasive breast cancer because its cancerous cells are still in the tissue of origination, however, it can increase the risk of developing invasive breast cancer (48). IDC has the highest incidence, at around 80%, and can invade the lymph nodes and other organs, whereas ILC has a lower incidence, at around 10-15%, and is slower growing than IDC (49). Breast cancer stage depends on whether the cancer has remained in the breast, spread to nearby tissues, or spread to distant tissues (48). Stage 0 is considered DCIS before it becomes invasive. In stage I, the cancer has started to invade normal surrounding breast tissue and is still small in size. By stage 2, the cancer has moved into the axillary lymph nodes 1 to 3, still within the breast region. In the next stage, it has moved to nodes 4 to 9 near the breast bone. Inflammatory Breast Cancer (IBC) sometimes occurs in this stage and involves the swelling and reddening of the breast. Finally, in stage 4, the cancer has spread beyond the breast into other organs such as distant lymph nodes, lungs, bones, liver, etc (48). The survival rate of stage 1 is around 100%, but by stage 4 it is reduced to 20-25% (50).

These breast cancer types can be further classified into molecular subtypes, including luminal A, luminal B, basal-like, HER2 overexpressing and normal breast-like (51). Luminal A and B cancers start in the luminal (inner) cells that line the mammary

ducts. Basal-like cancers lack a specific definition, but generally have the expression of proteins called basal cytokeratins (CK5/6, CK14, CK17) (51). Since each of these subtypes has a different prognosis, it is important to identify and classify them (52). These subtypes can be further classified as estrogen receptor (ER) positive or negative (+/-), progesterone receptor (PR) +/-, and human epidermal growth factor receptor 2 (HER2) +/- (53) (Figure 2.1). IDC breast cancers can come in different molecular subtypes (49). However, ILC breast cancer is generally ER+ and PR+, and luminal or normal-like (49,54,55). Often confused as a basal-like subtype, triple negative breast cancer (TNBC) is the most metastatic of these subtypes. In one study, 90.7% of TNBC patients had IDC histology and 82.5% had poorly differentiated cancers (56). It is the most aggressive and has the highest recurrence rate because of failure of available treatments (57). TNBC accounts for 10-20% of diagnosed breast cancers in women globally (51). TNBC subtypes have been found in younger patients and have a higher nuclear grade (evaluation of size and shape of nucleus) and histological grade (58). A cancer grade is measured by different factors such as nuclei prominence, variation and strangeness of morphology, and level of differentiation (59). Higher nuclear and histological grades are associated with poor prognosis (58). Consequently, TNBC has a lower 5-year survival rate (74.5% compared to 90.3% with all receptors positive) and a greater likelihood of recurrence outside the breast within the first 3 years after treatment (56,60,61). TNBC currently lacks targeted therapy and is treated by surgery or general chemotherapeutics (62).



**Figure 2.1. Simplified diagram of breast cancer molecular subtypes.** Breast cancer is a very complex disease to treat due to its heterogeneity. It is divided into 4 molecular subtypes, which are further subdivided into ER+/-, PR+/-, and HER2+/- (53).

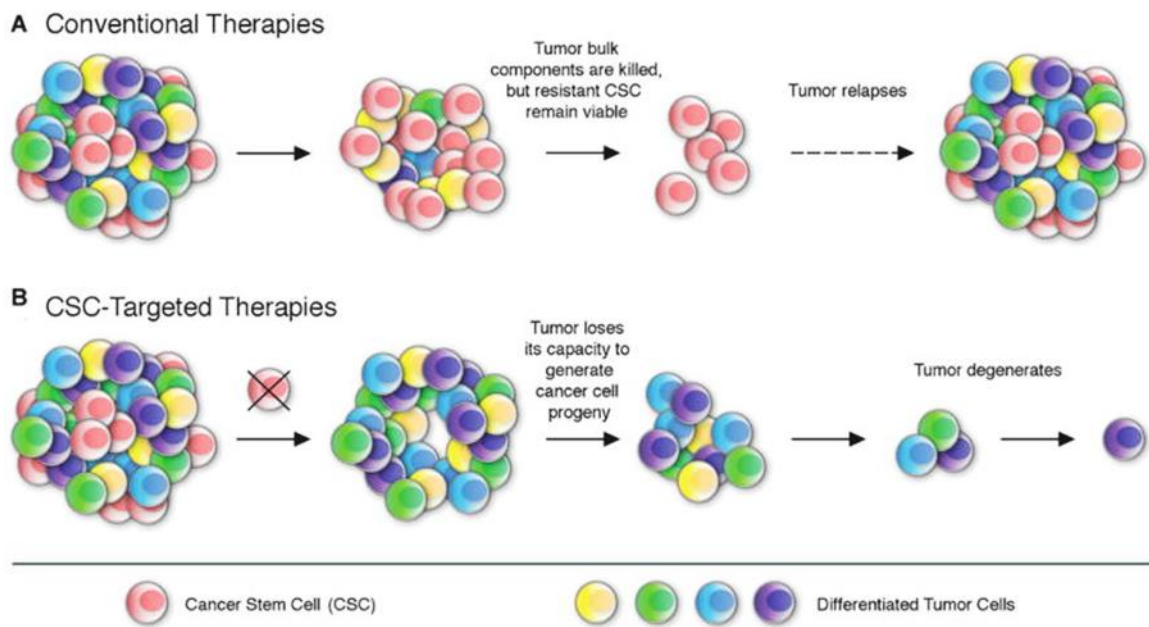
The previous AHCC, in vitro breast cancer study examined the triple negative breast cancer cell-line MDA-MB-231; an IDC adenocarcinoma (malignant tumor in glandular structures of the breast) (63,64). MDA-MB-231 has been used for breast cancer research for many years, with the earliest study recorded on PubMed occurring in 1978 (65). It is ideal for preclinical research since it is very aggressive in vitro as expected of a TNBC cell line (66,67). However, it is not as aggressive in vivo, unless directly added to the circulation system (68). MDA-MB-231 cells have a stellate morphology, a common characteristic of invasive cell lines (69). This makes it a useful cell line to examine invasive potential. Since breast cancer is heterogenous as a disease,

it is important to examine other breast cancer cell lines as well. Another commonly used human cell line is MCF-7. MCF-7 is an estrogen receptor positive, invasive breast ductal carcinoma, and therefore represents a different molecular subset of tumors from MDA-MB-231 (70). Further, this cell line differs in morphology, having round colony outlines (69). Estrogen receptor positive breast cancer represents 70-80% of breast cancers, making it very relevant to examine in studies (71). The 4T1 estrogen/IL-6 hormone-dependent, highly invasive and metastatic murine mammary carcinoma cell line (mouse version of TNBC) represents stage 4 cancer in humans and is a good model for studying metastatic potential in immunocompetent mice (72–74). Several recent studies have used these cell lines as models to examine cancer stem cell activity (75–79). These stem-like cells are part of a new and intriguing area of cancer research.

### **2.2.2 Cancer stem cells (CSCs)**

The pathological growth of tumors is maintained and driven by a small subpopulation of “stem-like” tumor cells, ensuring resistance to both chemotherapy and radiation (80–84) (Figure 2.2). This highly tumorigenic subset of cancer cells displays increased ability to self-renew, reproducing cancer heterogeneity, clonal tumor initiation capacity and long-term repopulation potential, and are designated Cancer Stem Cells (CSCs) (85). CSCs have been identified in solid tumors within the breast, colon, brain, prostate, pancreas, and hematopoietic system (86,87). In breast cancer, CSCs have the ability to grow as spheres or mammospheres, known as CSC phenotype CD44<sup>+</sup>/CD24<sup>-</sup> (88–90). In short, this means that BCSCs typically express the CD44 hyaluronic acid receptor, but not the CD24 heat stable antigen, both often expressed on tumor cells (91). In clinical settings, CSCs are shown to be responsible for relapse, therapy resistance, and

tumor recurrence (92). It may therefore be of interest to examine CSCs in TNBC, since it has the highest recurrence rate of the subtypes. One way to measure CSC growth parameters is through observing mammosphere growth. One disadvantage is that for MDA-MB-231 cells that have a stellate morphology (unlike MCF-7 and 4T1), cell viability drops if mammosphere go through a second passage (93) and, therefore, it only works for a single passage experiment. A single passage may not be enough to eliminate all regular cancer cells, so another procedure, such as flow cytometry will need to accompany it to examine BCSC populations.



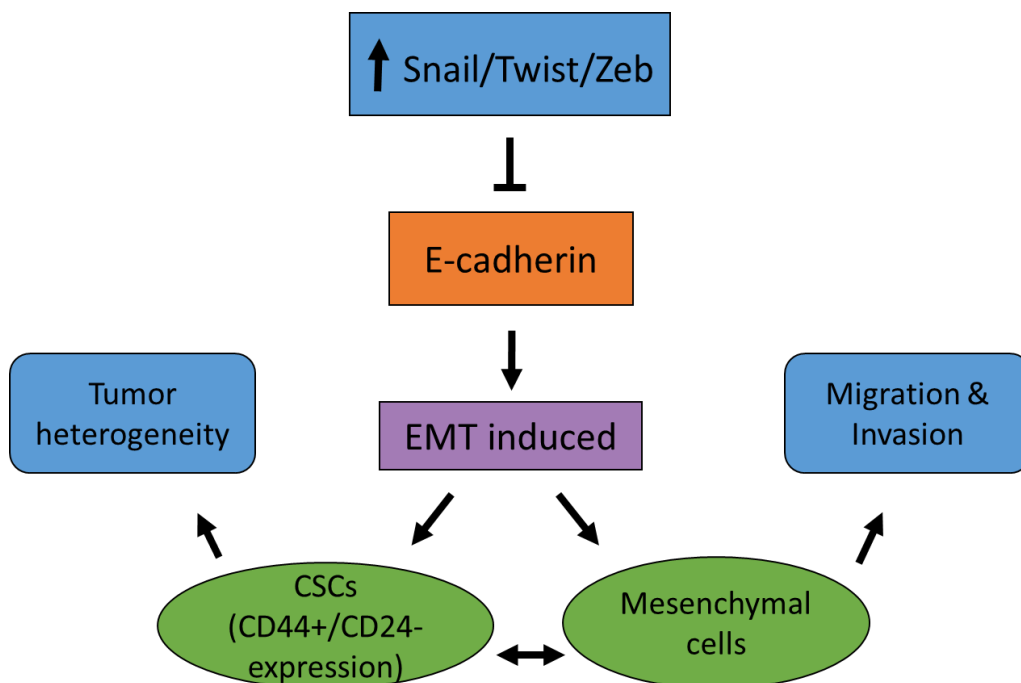
**Figure 2.2. CSCs escape chemotherapy treatment and lead to increased resistance and tumor relapse.** (a) Although current therapies target the bulk of the tumor, it is believed that a portion of self-renewing CSCs evade these treatments and regrow later on, leading to tumor relapse. (b) Therapies that target CSCs will inhibit the tumor from forming differentiated cells and prevent relapse. Figure taken from Schatton et al., 2009 (94).

### **2.2.3 Cancer stem cells, the epithelial-mesenchymal transition and metastasis: the involvement of key signaling pathways**

Cancer stem cells are suspected to be derived from the epithelial-mesenchymal transition (EMT), however their relationship is still not yet clear (95). The EMT is a two-way program of differentiation from epithelial cells to mesenchymal cells and vice versa, the mesenchymal-epithelial transition (MET) (96). This process is known to be involved in embryogenesis, wound healing and tumor metastasis (86,97). During the EMT, epithelial cells lose cell polarity and cell-cell adhesion, becoming migratory and invasive mesenchymal cells. This migration and invasion is permitted by transcription factors such as SNAIL, TWIST, and members of the ZEB families (98–101) (Figure 2.3). For instance, SNAIL1 works by directly repressing E-cadherin (E-cad), an epithelial adhesion molecule that acts as a tumor suppressor by maintaining the epithelial cell structure (102). During EMT, E-cad is replaced by mesenchymal markers such as N-cadherin, vimentin or fibronectin which allows the cells to detach from their basal membrane and thus promotes migration and invasion (103).

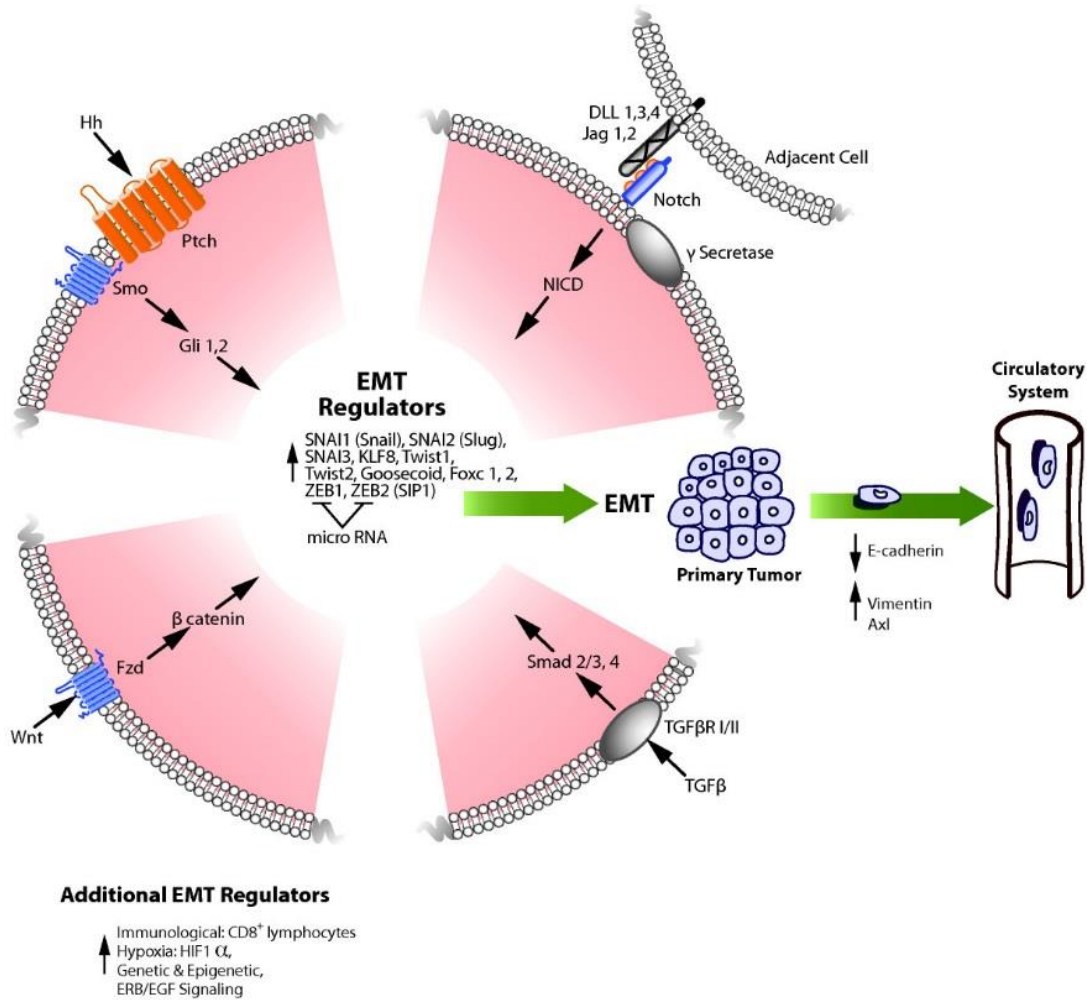
EMT may play a role in TNBC heterogeneity; a characteristic which makes treatment of TNBCs difficult (104). In one study, two clones of MDA-MB-231 cells were isolated, namely cells with fibroblast-like (F) and semi-epithelial (SE) morphology (105). It was discovered that F cells were more migratory and tumorigenic than the SE cells, which could therefore indicate that they are at a later stage in the EMT (105). Another study demonstrated that the induction of the EMT in immortalized human mammary epithelial cells (HMLEs) led to the expression of stem cell marker phenotype CD44<sup>high</sup>/CD24<sup>low</sup> cells (86). This implies that the EMT may be involved with CSCs,

although, again, there is still a dispute in the literature as to how the EMT, MET, and CSCs relate (95).



**Figure 2.3. EMT plays an important role in breast cancer heterogeneity and metastasis.** Transcription factors SNAIL, TWIST, and ZEB inhibit E-cadherin which induces EMT leading to tumor heterogeneity and migratory mesenchymal cells (98–103).

There are several pathways that connect the EMT to TNBC proliferation, differentiation, and invasion. These pathways include Notch, Wnt, Hedgehog, and TGF- $\beta$  signaling pathways (105–107) (Figure 2.4). Each pathway has separate mechanisms by which E-cad is influenced, and therefore, influences breast cancer cell heterogeneity, including the formation and maintenance of CSCs (105,108,109). Two of these pathways in particular, Notch and Wnt, are linked to our targets of interest.



**Figure 2.4 Regulatory pathways leading to the induction of EMT.** Notch, Wnt, Hedgehog, and TGF- $\beta$  pathways all induce the EMT pathway. Taken from Takebe et al., 2011 (110).

A functional Notch signaling pathway is necessary for stem cell proliferation (111). Deregulation of the pathway leads to cell proliferation, limits differentiation, and inhibits apoptosis in breast cancer cells (112–115). Important players in the pathway include NOTCH1 and NOTCH4, transmembrane Notch receptors. NOTCH1 is highly expressed in TNBC, including in MDA-MB-231 (106,116). Even more important in breast cancer, NOTCH4 activity is 8-fold higher in stem cell enriched populations than differentiated ones, is necessary for breast stem cell activity in vitro and influences tumor

growth in vivo (117). The ligands involved with the Notch signaling pathway include Delta-like ligands (DLLs) 1-4 and Jagged ligands (JAG) 1-2. A high expression of JAG1 and NOTCH1 correlated with poor breast cancer survival (118). Further, JAG1-NOTCH4 receptor activation prevented Notch signaling and breast cancer stem cell (BCSC) activity (119). The Notch pathway is activated by ligand binding to Notch receptors, where it is then cleaved and travels to the nucleus and communicates with transcription factors such as transforming growth factor-beta (TGF- $\beta$ ) (120). TGF- $\beta$  is a growth factor secreted by mesenchymal stem cells (MSCs) and is involved with breast cancer migration (121). This triggers the EMT in breast cancer, where SNAIL2 (SLUG), a direct target of the Notch pathway, downregulates E-cadherin (108). Furthermore, activation of Notch signaling increased the expression of SNAIL1 and TWIST1, which augmented the number of BCSCs, measured by mammosphere number (122). This evidence supports the important role of Notch signaling in EMT regulation of CSCs.

The Wnt/ $\beta$ -catenin pathway has been shown to be important for maintaining the pluripotency and differentiation of stem cells (123,124). In addition, the Wnt signaling pathway has been studied in MDA-MB-231 cells (105). Ligands in this pathway include 19 glycoproteins that bind the Frizzled (Fz) transmembrane receptor (125). This binding can lead to the accumulation of  $\beta$ -catenin in the nucleus, which transcriptionally activates targeted genes (110). The accumulation of  $\beta$ -catenin encourages the expression of Axin2, an intracellular protein that negatively regulates the pathway by causing the degradation of  $\beta$ -catenin (126). This then promotes the movement of glycogen synthase kinase-3 beta (GSK-3 $\beta$ ), a kinase, from nucleus to cytoplasm. GSK-3 $\beta$  phosphorylates  $\beta$ -catenin, causing its ubiquitination and degradation (110). GSK-3 $\beta$  also controls SNAIL1 stability

in breast cancer (127). As previously mentioned, SNAIL1 inhibits E-cad, as well as other cell-adhesion molecules such as occludin and claudin, thus promoting the EMT. Therefore, the Wnt pathway regulates EMT in breast cancer by the Axin2-GSK-3 $\beta$ -SNAIL1 axis.

#### **2.2.4 Cancer stem cells-How are they resistant?**

A number of chemotherapy agents are available for TNBC treatment, including taxanes (e.g. paclitaxel) and anthracyclines (e.g. doxorubicin) (62). Taxanes work by creating aberrant mitotic spindles that lead to apoptosis, whereas anthracyclines insert into DNA, inhibit topoisomerase that is involved in DNA coiling and form reactive oxygen species (62). There are also other forms of treatment such as radiation and immunotherapy. However, these treatments currently do not successfully target BCSCs. There are several mechanisms proposed for CSC resistance to chemotherapy, radiation, and immunotherapy (as well as the host immune system). Common theories of CSC chemoresistance include multidrug resistance (MDR) and tumor hypoxia, as well as immunotherapy and host tumor surveillance resistance by immune evasion.

MDR may be a way by which CSCs repel current therapies. Resistance may occur through the activation of DNA repair systems, alterations in drug targets, reduced uptake of drugs, detoxification by enzyme metabolism, p53 mutations, enhanced drug efflux by ABC transporters (e.g. multidrug resistance protein 1 (MDR-1) and P-glycoprotein (P-gp)), miRNA gene silencing, and epigenetic alterations (62,128–130). Taxanes and anthracyclins are substrates of P-gp and thus prone to drug efflux mechanisms (62). Furthermore, MDA-MB-231 cells high in transforming growth factor-

$\beta$  (TGF- $\beta$ ) ligands (part of the EMT) and with mesenchymal traits, are taxane-resistant (75). This indicates that CSCs have traits that resist current TNBC chemotherapeutics. Also, it is known that BCSCs have a CD44<sup>+</sup>/CD24<sup>-</sup> phenotype, and the CD44 receptor sends out survival and anti-apoptotic signals to CSCs, thus protecting them and allowing for MDR (131). Therefore, this phenotype may contribute to their particularly strong resistance to chemotherapeutics.

CSCs are known to be located in hypoxic niches, or specific areas that are low in oxygen, and hypoxia inducible factors (HIFs) maintain these stem cell niches by sustaining oxygen homeostasis (132,133). These areas prevent stem cells from depleting while preventing their over-proliferation throughout the body (133). As evidence, in vitro assays have shown that hematopoietic stem cells (HSCs) and MSCs preferred hypoxic conditions for growth and paracrine function (134–136). Chemotherapy may actually lead to an increase in CSCs by increasing HIF expression, thus promoting the maintenance of these niches (132). Also, a hypoxic environment encourages the aggressiveness of breast cancer. Breast tumor tissues have greater hypoxia in comparison to normal breast tissues, based on PO<sub>2</sub> measurements (137). Further, HIFs' higher expression predict early relapse and poor prognosis in breast cancer patients (138–140). This hypoxia has been associated with a poorer prognosis in a few different cancers (137). The hypoxia within tumors is due to poor oxygen delivery, because of cells being too distant from blood vessels and the abnormality of the intratumoral blood vessels (141). When particularly severe, this can lead to necrosis, or cell death, within solid tumors due to the lack of oxygen (142). The HIFs influence cancer metastasis through other important mechanisms, such as by targeting EMT components. For instance, HIF-

1 $\alpha$  increases SNAIL and TWIST expression, thus promoting EMT and cancer metastasis (143). Therefore, hypoxia and HIFs play a role in protecting CSCs from chemotherapy and lead to breast cancer metastasis.

CSCs can escape the host immune system and some immunotherapies by immune evasion; avoiding tumor surveillance through various mechanisms. Tumor surveillance is when the immune system actively searches and destroys tumor cells. It begins with acute inflammation, leading innate immune cells (macrophages, dendritic cells, natural killer cells, etc.) to enter into the tumor where they recognize pathogenesis and produce cytokines, causing dendritic cells to mature and go to activate T cells (144). These tumor-antigen specific CD4<sup>+</sup> and CD8<sup>+</sup> T Cells travel into the tumor to help kill the tumor cells (144). If successful, the tumor will be eradicated, otherwise, a subset of cells may evade the immune cells and live another day to revive the tumor mass (144). CSCs may be in this group of cells more commonly evading the immune system.

CSCs evade the immune system by differentially reacting to immune cells such as natural killer (NK) cells and T cells. This could also apply to some immunotherapies that promote T cell activity (145). To begin, CSCs may evade NK cells because they have reduced expression of NK ligands, MICA and MICB (146). Another proposed mechanism is that the CSCs use non-CSCs as a shield against cytotoxic T lymphocytes (CTLs) by promoting resource competition, such as for glucose, between non-CSCs and CTLs (147). Also, protein pathways can modulate CTL activity in CSCs. For example, the transcription factor Nanog has been shown to be important for immune evasion by CSCs. Nanog is induced by hypoxia, common in a CSC environment, and was found to

modulate regulatory T cells, macrophages, and CTLs (148). Further, CSCs with a Nanog<sup>+</sup> phenotype were more likely to evade CTLs (149). Since Nanog<sup>+</sup> stem-like cells are missed by the CTLs, CSCs will continue to grow within the tumor, increasing immune resistance (149). This form of positive feedback loop is likely to be the case for some other protein pathways that help in immune evasion.

### **2.2.5 Tenascin C and cancer stem cell resistance**

Another protein, the oncogenic Tenascin C (TNC), is an extracellular matrix protein that is expressed in most solid tumors (and secreted by them), downregulates tumor suppressors, and stimulates oncogenic signaling (150–152). TNC is a very promising target for BCSC research. Interestingly, TNC has been shown to regulate the EMT and thus influence CSCs (153,154). Although it is almost absent in normal adult tissue, it has been shown to be highly expressed during tissue repair and embryonic development, as well as in chronic inflammation and cancer (150,155–158). All of these processes have been associated to the EMT. In fact, TNC proteins are found at EMT sites in the embryo, as well as in developing tissues such as bones, tendons, and cartilage (159–161). It has also been shown to be necessary for injury-induced EMT in the lens epithelium of the eyes (153). There are several proposed mechanisms for TNC action, including the stimulation of pro-inflammatory cytokines and oncogenic signaling molecules to control cell migration, proliferation, and signaling, all of which play important roles in cancer (155).

More specifically, TNC promotes the expression of stem cell signaling components involved in the Notch and Wnt signaling pathways, including musashi

homolog 1 (MSI1) and leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5) (162). TNC prevents the inhibition of the Notch pathway by signal transducer and activator of transcription factor 5 (STAT5), as well as promotes the expression of Wnt target gene LGR5 (162). Furthermore, TNC is needed for the activation of the Wnt/ $\beta$ -catenin signaling pathway in the stem cell niches, allowing for the maintenance of the stem cell pool (163). Additionally, the cytokines tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interferon- $\gamma$  (IFN- $\gamma$ ) have been shown to positively regulate the expression of TNC in vivo (164).

### **Tenascin C and Multidrug Resistance**

One study suggested that TNC may be involved in CSC MDR. The researchers demonstrated that TNC knockdown (in WM3734 cells) prevented melanoma progression by lowering the CSC side population that was positive for ABC transporter ABCB5 (165). This lowered the resistance of the melanoma cells to doxorubicin, possibly because of the reduction in ABC transporters (165). This study further supports that TNC plays a role in CSC maintenance.

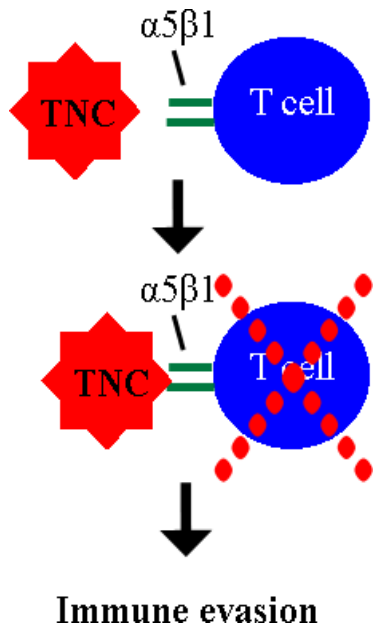
### **Tenascin C and Hypoxia-inducible factor 1-alpha**

Since TNC is involved with CSC side population maintenance, it is possible that it is somehow involved in maintaining hypoxic conditions. HIF-1 $\alpha$  was found to positively correlate with TNC expression in thyroid cancer and HIF-2 $\alpha$  correlated with TNC expression in bladder cancer tissue samples (166,167). As mentioned previously, these HIFs are important in maintaining stem cell niches and encouraging EMT and

metastasis (132,133,143). This indicates one more mechanism by which TNC may be involved with CSC maintenance and cancer metastasis.

### **Tenascin C and immune evasion**

In cancer settings, TNC has been shown to be involved with immune evasion. Interestingly, CSCs have been shown to avoid immune surveillance through the TNC- $\alpha 5\beta 1$  axis (168). TNC interacts with T cells through the  $\alpha 5\beta 1$  integrin on the surface of the T cells (162,168,169) (Figure 2.4). Its mechanisms involve preventing the reorganization of the actin-based cytoskeleton needed for proper T cell activation, proliferation, and cytokine production (169). Additionally, a study looking at rheumatoid arthritis found that TNC interacts with the integrin  $\alpha 9\beta 1$  and regulates IL-17 secreting helper T cells (Th17), enhancing the production of cytokines by conventional Dendritic Cells (cDCs) and macrophages with the help of TLR4 and 2 signaling (170). TNC was found to be negatively associated with T cell migration (171). Another study found that the TNC  $\alpha 9\beta 1$  integrin receptor was important in regulating thymic T cell progenitor migration, with a lack of TNC leading to a greater number of mature T cells circulating the lymphoid system (171). CTLs are a promising target in TNBC since it was found that CTL tumor infiltration capacity of TNBC positively correlated with a good outcome of the disease, whereas if the CTLs were marginalized to the stroma, this was an indication of a poor outcome (172). It is clear from these previous findings that TNC could be a key target for cancer products such as AHCC, since it plays a central role in many cancers and CSC-related pathways that promote immune and chemoresistance.



**Figure 2.5. TNC prevents T cell activity through integrin  $\alpha5\beta1$ .** TNC protects CSCs from the immune system by preventing T cell activation, proliferation, and cytokine production (169).

## 2.3 Epigenetics and the impact of diet

One way to target CSC resistance pathways may be through epigenetic mechanisms. Epigenetic mechanisms appear to play a fundamental role in cancer establishment and progression of tumors. Their deregulation has been reported at multiple levels, including DNA methylation, histone modification, and microRNA expression (173–185). DNA methylation on CpG islands can determine which kind of histone modification will occur at the gene promoter. Histone modifications induce factors to come and cause wrapping of the DNA around the histone bodies, which blocks transcription of particular genes or, alternatively, chromatin remodeling factors will remove histone bodies to allow access to the DNA (30). These alterations work to either increase protein activity or limit it, changing important molecular pathways in the body.

In contrast to genetic mechanisms, epigenetic changes are reversible and, therefore, susceptible as targets for intervention (85,179). Moreover, growing evidence supports an early deregulation of epigenetic profiles during breast carcinogenesis (186–188). In this sense, various dietary regimes are suspected to modulate susceptibility to breast cancer by altering normal epigenetic states as well as reversing oncogene activation or silencing tumor suppressors genes (189). Epigenetics influenced by the diet is known as nutritional epigenetics. Numerous studies suggest that nutritional compounds have epigenetic targets in CSCs (189) (Table 2.1).

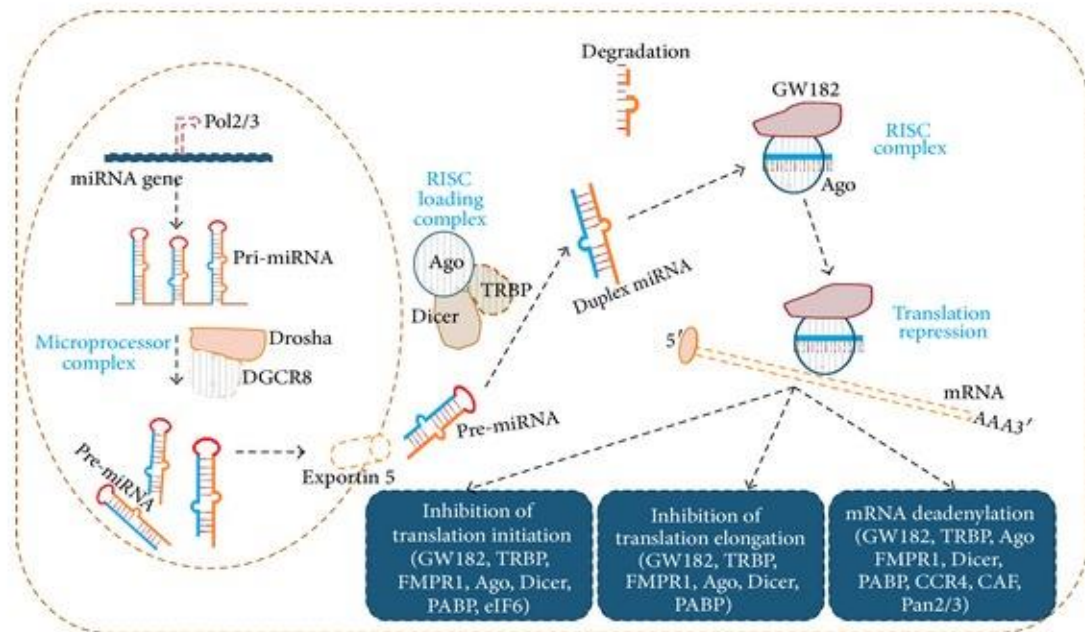
**Table 2.1.** Nutritional compounds with epigenetic targets in CSCs.

Compound	Targets/Effects
<b>Polyphenols</b>	<ul style="list-style-type: none"> <li>• Seaweed polyphenols caused dose-dependent reduction in cell viability of pancreatic CSCs and influenced E-cadherin, among other targets (190).</li> <li>• Tea polyphenols limit liver cancer by modulating Wnt and Hedgehog pathways (191).</li> <li>• Tea polyphenols caused apoptosis and prevented growth of inflammatory breast CSCs by altering gene expression (192).</li> </ul>
<b>Soy isoflavone genisten</b>	<ul style="list-style-type: none"> <li>• Repressed mammosphere formation in breast cancer cells and altered gene expression (193).</li> </ul>
<b>Curcumin</b>	<ul style="list-style-type: none"> <li>• Curcumin suppressed ABCG2 expression in BCSCs and improved their sensitivity to chemotherapeutics (194).</li> <li>• Curcumin inhibited Wnt signaling in BCSCs (195).</li> </ul>
<b>Resveratrol</b>	<ul style="list-style-type: none"> <li>• Downregulated pLKB1 (liver kinase) that is involved in leukemia stem cell senescence (196)</li> <li>• Killed ovarian CSCs and caused loss of self-renewal capability (197).</li> </ul>

We postulate that AHCC may target CSCs through epigenetic mechanisms, including through regulating microRNA expression. Recent studies have shown that natural agents, including resveratrol, could alter miRNA expression profiles (198), leading to reversal of the EMT, or enhancement of efficacy of conventional cancer therapeutics.

### **2.3.1 MicroRNA mechanisms**

MicroRNAs (miRNAs) represent a subset of endogenous small non-coding RNAs, approximately 21-25 nucleotides in length, with a striking ability to control the expression of approximately one third of the human genome (199). Pre-miRNAs are synthesized from primary miRNAs (pri-miRNAs) by the type 2 RNAIII enzyme Drosha in the nucleus, are brought to the cytoplasm by exportin-5, processed by the RNAIII-like enzyme Dicer into mature miRNAs, and finally are bound to the Argonaute (Ago) protein to create the RNA-induced silencing complex (RISC) (200). In a pathway commonly known as RNA interference (RNAi), RISC recognizes its complementary mRNA transcript and the endonuclease Slicer cleaves it, leading to its degradation (201). mRNA degradation occurs if it is a nearly perfectly complement of the RISC (201). mRNA degradation reduces the number of transcripts available for translation into proteins, therefore lowering protein expression (201) (Figure 2.5). MiRNAs have many protein targets and proteins are each targeted by many different miRNAs. This is an important control mechanism for diseases such as breast cancer.

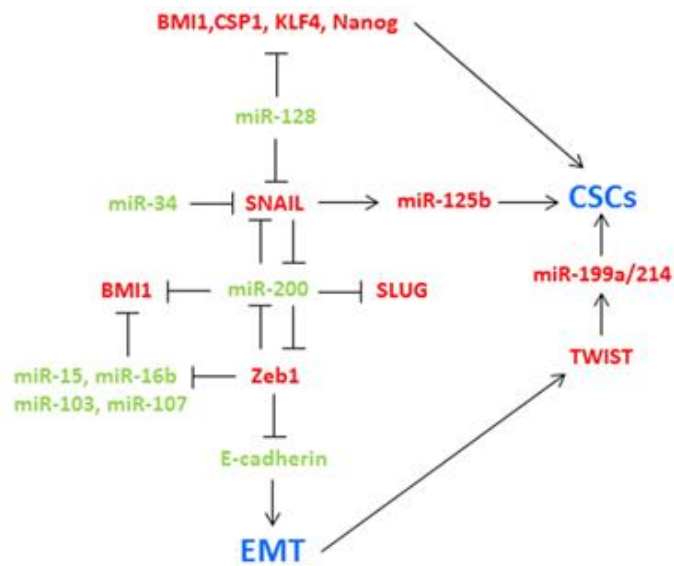


**Figure 2.6. MicroRNAs work at the transcriptional level to repress protein expression.** MicroRNAs start off in the nucleus where they are processed from pri-miRNAs to pre-miRNAs. Exportin then carries the pre-miRNAs out to the cytoplasm where they are bound by RISC and become mature miRNAs. Based on their sequence, they can inhibit translation initiation, elongation, or lead to mRNA degradation. Figure taken from Vimalraj et al., 2013 (202).

### 2.3.2 MicroRNAs in Breast cancer

MicroRNAs are often over-expressed or down-regulated in a number of malignancies (203) and some can also function as tumor suppressors or as oncogenes (204). They participate in the regulation of tumor cell growth, invasion, drug resistance, and metastasis. One study found a panel of 38 miRNAs to be differentially expressed between molecular subtypes of breast cancer and another found 59 differentially expressed miRNAs in breast cancer tissues in comparison to healthy tissues (205,206). Among aberrantly expressed miRNAs, miR-145 and miR-125b were reported to be significantly down-regulated, whereas miR-21 and miR-103 were up-regulated (207). In addition, high expression of miR-34a expression is modulated by p53 (the most

frequently altered gene in human cancers) and is associated with a lower risk of recurrence or death due to breast cancer (208). High expression of miR-210 is associated with poor survival of breast cancer patients (2). miR-21 has been associated with tumor proliferation and metastasis, and when antagonized, reverses EMT and CSC phenotype (209). It has been associated with breast cancer metastasis and poor prognosis (210). miR-365 is important in certain breast cancer-related pathways, such as the IL-6 pathway, which is associated with breast cancer metastasis (211). In summary, many miRNAs have important roles in EMT and CSC pathways, and in breast cancer progression (Figure 2.6).



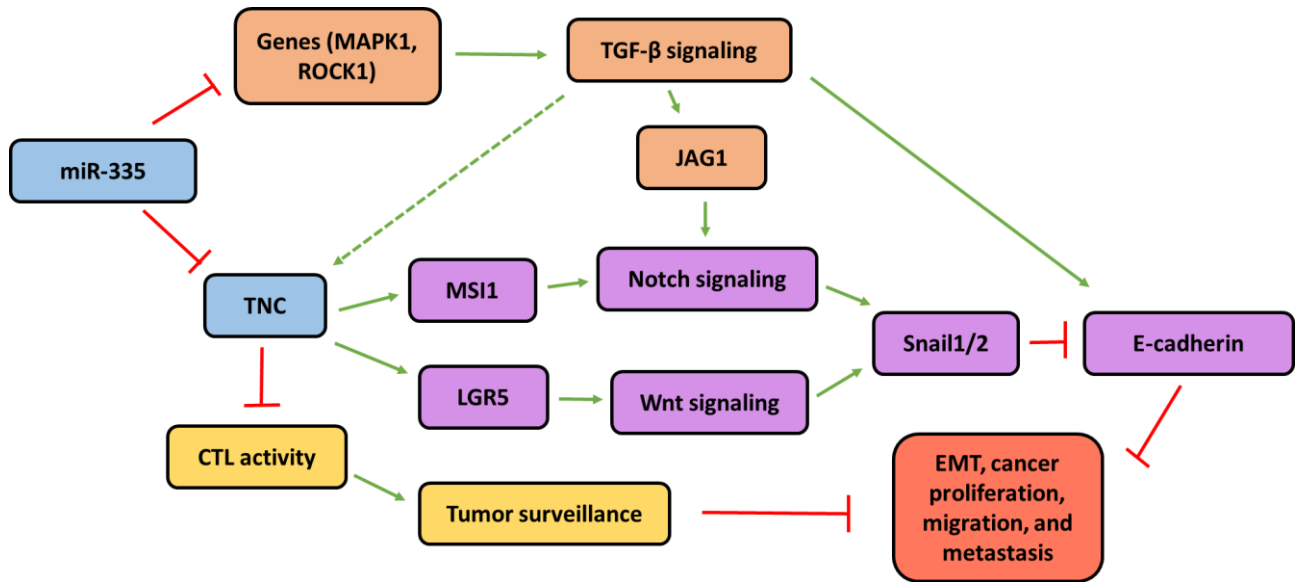
**Figure 2.7. MicroRNAs play an important role in regulating EMT and CSC pathways.** MicroRNAs are integral to CSC pathways and have many targets in EMT pathways, making them valuable targets to study in the lab. Terms in red are upregulated and those in green are downregulated. Figure taken from Garofalo and Croce, 2015 (212).

In particular, miR-335 is down-regulated in breast cancer and has been shown to suppress tumor invasion and metastasis (213). miR-335 has been shown to be more down-regulated in tumors of breast cancer patients with breast cancer gene (BRCA) mutations in comparison to those in patients without (214). Especially interesting, miR-335 was found to be down-regulated in CSCs and to inhibit CSC growth (215). Furthermore, miR-335 has been shown to negatively regulate HIF-1 $\alpha$  in a murine cell line and rat animal model, further connecting it with CSC growth prevention (216). It is possible that miR-335 regulates proteins such as TNC through important CSC-related pathways (217,218).

### **2.3.3 miR-335 protein targets and pathways**

miR-335 has also been implicated in modulating several protein pathways. One study found that miR-335 negatively regulated the c-Met protein, which is involved in cell migration and invasion (213). Another study found that miR-335 negatively regulated the paired box 6 gene (PAX-6) expression in MCF-7 cells and thus caused cell cycle arrest in the G1 phase, preventing cell migration (219). Additionally, miR-335 is connected to p53 through regulating the expression of the retinoblastoma family (Rb1) protein, and works together with p53 in a positive feedback loop to drive cell cycle arrest (220). Further, miR-335 has been shown to suppress metastasis and migration by targeting transcription factor SOX4 and extracellular matrix protein Tenascin C (TNC) (218). More specifically, upregulation of miR-335 has been shown to downregulate TNC by inhibiting its gene expression in MDA-MB-231 cells, which prevented breast cancer cell migration and invasiveness (217,218). It was also shown to be involved with the ligand JAG1 from the Notch pathway. When miR-335 was knocked down along with

miR-21 and miR-153, JAG1 significantly increased (221). Furthermore, another study showed that the Wnt signaling pathway upregulated miR-335 in human mesenchymal stem cells (222). These studies demonstrate that miR-335 is a promising target in BCSCs since it is involved with TNC and EMT pathways such as Notch and Wnt (Figure 2.7).



**Figure 2.8. miR-335 plays a pivotal role in regulating EMT pathways through TNC and other targets, therefore preventing cancer metastasis.** In one pathway, miR-335 inhibits genes controlling the TGF- $\beta$  signaling pathway, which has been proposed (dotted line) to influence TNC activity. In another pathway, miR-335 inhibits TNC protein expression, which leads to the inhibition of EMT inducing pathways such as Notch and Wnt and the induction of CTL activity and tumor surveillance. Through these pathways, miR-335 prevents EMT and CSC properties, cancer cell proliferation and migration, and cancer metastasis (108,122,127,162,163,169,218,223–227). Blue indicates the targets of interest in this study, orange indicates the TGF- $\beta$  signaling pathway, yellow indicates the immune pathway, purple indicates the Notch and Wnt pathways, and pink indicates the effect of these pathways.

## 2.4 Hypothesis and objectives

The accumulated evidence regarding anti-tumoral activities of AHCC in the literature indicates that it is involved in the control of inflammatory pathways in cancer progression. Our hypothesis is that AHCC modulates a specific subset of miRNAs that regulate the expression of oncogenic factors affecting CSC survival/stemness pathways. The purpose of this project is to study AHCC modulatory effects on CSCs in an in vitro, ex vivo, and in vivo setting. The specific objectives are to:

**Objective 1:** Analyze the role of AHCC in mammosphere development and tumorigenesis.

- Study the effects of AHCC on mammosphere growth and cell motility in different cell lines (MDA-MB-231, MCF-7, 4T1).
- Validate the effect of AHCC on CSC growth in ex vivo and in vivo settings with Balb/c mice.

**Objective 2:** Evaluate the microRNA signature of AHCC in cell culture

- Establish the impact of AHCC in creating a distinct miRNA signature in MDA-MB-231 by miRNA profiling and miRNAome study.
- Validate the differentially expressed miRNAs by RT2-qPCR in mammospheres and in vivo.
- Determine by transfection assays the role of tumor suppressor miR-335 and study the protein target Tenascin C.

## Chapter 3 : Manuscript for publication

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**Keywords:** breast cancer, cancer stem cell, AHCC, microRNA, chemoprevention

Prepared for submission to Breast Cancer Research and Treatment Journal

### **3.1 Contributions**

**Émilie Graham:** cell culture, RT2-qPCR, mouse gavage and tissue processing, western blot, cell motility assays, writing, editing

**Jean-François Mallet:** mentor, trainer, lab manager, mice injections and surgery, editing

**Majed Jambi:** flow cytometry analyses, editing

**Hiroshi Nishioka:** study design

**Kohei Homma:** study design

**Chantal Matar:** supervisor, study design, editing

## 3.2 Abstract

**Purpose:** Many breast cancer patients are using natural compounds in their battle against breast cancer. Active Hexose Correlated Compound (AHCC®) is a cultured mushroom mycelium extract shown to favorably modulate the immune system and alleviate cancer burden. Breast Cancer Stem cells (BCSCs) are a subset of highly tumorigenic cancer cells that are thought to be responsible for recurrence. BCSCs can be epigenetically regulated by microRNAs (miRNAs). We hypothesized that AHCC may influence BCSCs by modulating tumor-suppressor or oncogenic miRNAs. **Methods:** BCSCs were isolated in the form of mammospheres from MDA-MB-231, MCF-7, and 4T1 cells, exposed to AHCC in both regular and primary culture from Balb/c mice, and analyzed by visual counting and flow cytometry. Cell motility was also observed in MDA-MB-231 cells. Following, we performed profiling and RT2-qPCR to determine AHCC influence on miRNAs in MDA-MB-231 mammospheres. Additionally, Balb/c mice were orally gavaged with AHCC and tumor growth parameters and miR-335 expression were analyzed. Finally, we transfected MDA-MB-231 cells with miR-335 and performed western blotting analyses. **Results:** We demonstrated that AHCC reduced mammosphere growth in three cell lines and in primary culture, prevented cell migration, and upregulated miR-335 expression in MDA-MB-231 cells and mouse tumor samples. Among the differentially regulated miRNAs in BCSCs, we focused on tumor suppressor miR-335, known to target extracellular matrix protein, Tenascin C (TNC). TNC is involved in CSC immune evasion pathways. In MDA-MB-231, inhibition of miR-335 increased TNC protein expression. **Conclusions:** These results support that AHCC prevents BCSC growth, partly by targeting miRNA pathways.

## **Abbreviations**

**AHCC:** Active Hexose Correlated Compound

**CAM:** Complementary and alternative medicine

**BCSC:** Breast cancer stem cell

**CSC:** Cancer stem cell

**EMT:** Epithelial-mesenchymal transition

**NK:** Natural killer cell

**TNC:** Tenascin C

**TNBC:** Triple negative breast cancer

## **3.3 Introduction**

Currently, there is considerable interest in nutritional interventions and natural compounds for targeting key deregulated niche signaling pathways in tumor development. For example, a dietary intervention showed that a few months of Mediterranean diet was sufficient to positively change the metabolic/endocrine characteristics of breast cancer survivors (228). More specifically, the naturally derived product Active Hexose Correlated Compound (AHCC®), from the culture of Basidiomycete mushroom extract, has been reported to exert immunoprotective effects against many types of cancer, including liver, breast, colon and prostate (20,22,43,229). It is thought that the active component in AHCC are its acylated  $\alpha$ -1,4 glucans (230). Further, it has been shown to increase the activity of immune cells in clinical studies (38,39) and enhance the antitumor effects of certain chemotherapy drugs in (43) and have anti-inflammatory effects in animal studies (44).

The pathological growth of tumors is maintained and driven by a small subpopulation of “stem-like” tumor cells, ensuring resistance to both chemotherapy and radiation (80–84). This highly tumorigenic subset of breast cancer cells display increased ability to self-renew, generate breast cancer heterogeneity, and are designated Cancer Stem Cells (CSCs) (85). In breast cancer, CSCs can be isolated by the CD44<sup>+</sup>/CD24<sup>-</sup> phenotype and by their ability to grow as spheres known as mammospheres (88–90). CSCs are believed to be connected to the epithelial-mesenchymal transition (EMT), which is associated with metastasis as it allows cells to migrate and invade nearby tissues and enter the bloodstream (209).

The oncogenic Tenascin C (TNC), is an extracellular matrix protein that is expressed in breast cancer cells and is negatively associated with T cell migration (162,171). It has been shown to be highly expressed during tissue repair and embryonic development, as well as in chronic inflammation and cancer (150,155–158). TNC promotes the expression of stem cell signaling components involved in EMT pathways and is needed for the activation of the Wnt/ $\beta$ -catenin signaling pathway in the stem cell niches, allowing for the maintenance of the stem cell pool (162,163). CSCs have been shown to avoid immune surveillance through the TNC- $\alpha$ 5 $\beta$ 1 axis (168). This immune evasion may be one explanation for CSC survival and tumor recurrence.

CSCs are subject to epigenetic regulation, particularly by the expression of microRNAs (miRNAs); small, noncoding RNAs that have emerged as critical regulators of CSC functions in cancer initiation, therapy resistance and metastasis (231). Recent studies have shown that natural agents can alter miRNA expression profiles (198).

miRNAs are often over-expressed or down-regulated in a number of malignancies (203) and some can also function as tumor suppressors or as oncogenes (204). In particular, miR-335 is down-regulated in breast cancer, especially in patients with BRCA mutations (214). It has also been implicated in modulating several protein pathways (202,213,219,220), including suppressing metastasis and migration by inhibiting TNC expression (202). Additionally, miR-335 was found to be down-regulated in CSCs and to inhibit CSC growth (215). These studies demonstrate that miR-335 is a promising target in breast cancer stem cells.

Therefore, we propose that AHCC targets CSCs through epigenetic mechanisms, such as through changing the expression of microRNAs, such as miR-335, and indirectly inhibiting Tenascin C protein expression. We investigated these effects in three different cell lines, with a focus on a TNBC cell line, and in Balb/c mice.

## **3.4 Methods**

### **3.4.1 AHCC preparation**

AHCC was obtained from the Amino Up Chemical Co., Ltd. (Sapporo, Japan). We prepared 40mg/ml AHCC in DMEM-F12 and filtered it through a 0.22µm Millex-GV filter.

### **3.4.2 Cell culture and mammosphere growth**

MDA-MB-231 and 4T1 cell lines were cultured in RPMI 1640 (1X) and MCF-7 cells were cultured in DMEM (1X) (Gibco, Grand Island, NY, USA). All cells were incubated at 37°C and 5% CO<sub>2</sub>. Adherent cells were detached by trypsin and single cells were counted using the Countess automated cell counter (Invitrogen, Burlington, ON).

Afterwards, the cells were plated on Costar ultra-low attachment plates (Corning, St. Laurent, QC) in spheroid medium. Spheroid medium consists of DMEM-F12 (Invitrogen) combined with 100mM Sodium pyruvate, 250mM L-glutamine (Sigma Aldrich, Oakville, ON), 100ug/mL hydrocortisone (Sigma Aldrich), 1000x streptomycin-penicillin (Sigma Aldrich), 20ug/mL bFGF, 20ug/mL EGF, and 10mg/mL insulin.  $2 \times 10^4$  cells/ml were plated in 96-well ultra-low attachment plates and  $2 \times 10^5$  cells/ml (for a 24h extraction) or  $3 \times 10^5$  cells/ml (for a 5h extraction) were plated in 6-well ultra-low attachment plates. Mammospheres were exposed to 0-4mg/ml AHCC in treatment group. Mammospheres were counted in the 96-well ultra-low attachment plates by light microscopy and miRNA were extracted from mammospheres in 6-well ultra-low attachment plates after 5 hours. At least three samples were used for analysis for each cell line.

### **3.4.3 Flow cytometry**

Three samples of mammospheres from MDA-MB-231 were collected after 24h, washed with phosphate-buffered saline (PBS) and then enzymatically dissociated with 0.05% trypsin/0.25% EDTA into single cell suspension. Combinations of monoclonal antibodies against human cell, CD44-APC (BD Biosciences) and CD24-PE/CY7 (eBioscience), were added to the cell suspension at concentrations recommended by the manufacturer and incubated at 4°C in the dark for 30 to 40 minutes. For all mammospheres, labeled cells were washed in PBS to eliminate unbound antibody, and the flow cytometry was analyzed on a BeckmanCoulter MoFlo XDP (San Francisco, CA, USA). Dead cells were eliminated by using the viability dye DAPI. Side scatter and

forward scatter profiles were used to eliminate cell doublets. A minimum of 10,000 events were recorded for each sample.

#### **3.4.4 Cell viability and proliferation**

MDA-MB-231 cells were grown in a 96-well plate at a concentration of  $2 \times 10^5$  cells/ml in RPMI medium with FBS, streptomycin and penicillin. The cells were then exposed to concentrations of 0-8mg/ml AHCC for 24 hours. The MTT-based In Vitro Toxicology Assay Kit (Sigma-Aldrich) and protocol was followed with an incubation period of 4 hours. Three samples were used for analysis.

#### **3.4.5 Cell motility**

MDA-MB-231 cells were plated in a 6-well Corning tissue culture treated plate at a concentration of  $3 \times 10^5$  cells/ml and exposed to 0-4mg/ml of AHCC for 24 hours at 37°C and 5% CO<sub>2</sub>. A scratch was then made with a 1000ul pipette tip and photographs were taken at 0 hours, 24 hours, and 48 hours or 72 hours. Three samples were used for analysis.

#### **3.4.6 MicroRNA profiling**

MDA-MB-231 mammospheres were extracted 24 hours after plating. The two treatment group samples were exposed to 4mg/ml of AHCC for 24 hours and the two control group samples were given an equal volume of medium. Sample purity and integrity were verified with a NanoDrop 2000 (Thermo Scientific, Waltham, MA, USA) and Agilent 2100 BioAnalyzer (Bio-Rad, Hercules, CA, USA), respectively. Microarray analysis was completed by an Affymetrix GeneChip® miRNA 3.0 Array

### **3.4.7 Real-time reverse quantitative PCR**

After 5 hours of exposure to AHCC or control conditions, cancer stem cells were extracted using the miRNeasy mini kit (Qiagen, Toronto, ON). Then, the samples underwent a reverse transcription reaction to form cDNA by using individual probes. The cDNA was synthesized by Moloney Murine Leukemia Virus (MMLV) Reverse Transcriptase (Invitrogen). The expression of snRNA-U6, and target miR-335, was measured by RT2-qPCR using Taqman probes (Applied Biosystems, Burlington, ON) and a FastStart Taq Polymerase (Roche, Mississauga, ON) in a CFX96 machine (Bio-Rad). Three samples were used for analysis.

### **3.4.8 Transfection**

MDA-MB-231 cells were allowed to grow to 30% confluence in RPMI medium with FBS and antibiotics in 15ml flasks. The cells were then incubated with lipofectamine (Life Technologies, Burlington, ON) and the targets: non-coding control, miR-335 mimic, and miR-335 inhibitor (Ambion, ThermoFisher Scientific) for 48 hours. After incubation, a passage was completed and cells were plated in regular 6-well plates or ultra-low attachment. MicroRNA were then extracted, reverse transcribed, and analyzed by RT2-qPCR.

### **3.4.9 Protein analysis**

Three samples of MDA-MB-231 cells were transfected for 48 hours in a 6 well attachment plate and extracted. Cells were washed with PBS on ice then mixed with 300ul Pierce® RIPA buffer (Thermo Scientific) combined with the Halt™ Protease and Phosphatase Inhibitor Cocktail (100X) (Thermo Scientific). Cells were then scraped off

the plate and protein samples were centrifuged to remove the outer membrane. Protein concentrations were quantified by the BCA Protein Assay Kit (Pierce). Samples were prepared in Laemmli sample buffer, heated for 5 min at 95°C and loaded on precast Bolt® Bis-Tris Plus Gels (Invitrogen). The electrophoresis was carried in MES buffer (Invitrogen) at 200 volts in a Mini Gel Tank (Life Technologies). The gels were transferred to Immobilon-p50 PVDF membranes in a Trans-Blot Cell (Bio-Rad). The membranes were exposed to primary antibodies Anti-Tenascin C at a dilution of 1:2500 (Abcam ab108930, Cambridge, United Kingdom). The secondary antibody was a goat anti-rabbit used as a 1:10000 dilution (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Membranes were archived with a VersaDoc (Bio-Rad) and the intensity of each band was measured using Quantity One software (Bio-Rad).

#### **3.4.10 Mouse handling and feeding procedures**

Thirty-one, six to eight week old Balb/c mice weighing between 18-20g were obtained from Charles River (Montreal, QC, Canada). The mice were handled based on guidelines required by the Canadian Council on Animal Care and the experimental design was approved by the University of Ottawa Animal Care Committee under protocol ME-316. Mice were housed in plastic micro-insulators in a controlled atmosphere (temperature:  $22 \pm 2$  °C; humidity:  $55 \pm 2$  %) on a 12-h light/dark cycle and fed a conventional balanced diet (2018 Teklad Global 18 % Protein Rodent diet, Harlan Laboratories Inc, Madison, Wisconsin, USA) and water ad libitum. Eleven mice were gavaged with AHCC in 1% sucrose in water solution (1g/kg/day) for two week before tumor injection and 19-23 days following until sacrifice. The ten control group mice were gavaged with 1% sucrose in water solution instead. The ten mice saved for ex vivo studies were

examined periodically following injection for stress that may have been caused by tumor injection. All mice were injected with 1400 4T1 cells in the right abdominal mammary gland.

#### **3.4.11 Tumor extraction, measurement and mammosphere plating**

Tumors were extracted based on the protocol described by Pulaski and Ostrand-Rosenberg (232) with a few minor changes. Tumors were weighed on a scale and measured with a caliper. For the ex vivo experiment, approximately 0.05 g of each tumor was minced and dissociated in RPMI-1640 media containing 300 U/ml collagenase (Sigma) at 37°C for 2 hours. Cells were sieved sequentially through a 40 µm cell strainer (BD Biosciences, San Diego, CA, USA) to obtain a single cell suspension. These cells were then counted using a Countess automated cell counter (Invitrogen, Burlington, ON) to determine concentration and viability. The cells were plated in ultralow attachment 96-well plates (Corning) at a concentration of  $2 \times 10^4$  cells/ml, in DMEM-F12 (Invitrogen), supplemented with 10 ng/ml EGF, 20 ng/ml bFGF, 5 µg/ml insulin, 1 mM sodium pyruvate, 0.5 µg/ml hydrocortisone, and penicillin/streptomycin (0.05 mg/mL) (Sigma). The mammospheres were exposed to concentrations of 0, 2, and 4 mg/ml of AHCC and examined at 24, 48, and 72 hours.

#### **3.4.12 Statistical analysis**

Mammosphere growth in vitro and RT2-qPCR results were analyzed by one-way ANOVA and post-hoc Tukey test, and ex vivo mammosphere growth and cell motility by two-way ANOVA on GraphPad Prism 5 (La Jolla, CA, USA). Flow cytometry results were analyzed with Kaluza 1.3 software (Beckman Coulter Inc., Montreal, QC). The cell

motility assay was analyzed with TScratch software (CSE Lab, Zurich, Switzerland). Profiling results were analyzed with Mev 4.8.1 software, BRB Arraytools 4.4.0. The data was normalized using Robust Multichip Average (RMA) (233). The statistical analysis was done by a two-class unpaired SAM 1.0. Western blot analyses were conducted using Biorad Image Lab 5.2.1. Tumor volume and mass were analyzed by a student's t-test on GraphPad Prism 5.

## **3.5 Results**

### **3.5.1 Mammospheres**

A common procedure used to examine BCSC growth is through the isolation and culturing of mammospheres, small congregations of BCSCs suspended in the media (93). Mammosphere growth was observed for 24 hours after exposure to AHCC in three cell lines: MDA-MB-231, 4T1, and MCF-7. A dose dependent effect was observed in all cell lines (Fig 1a-d). All ANOVA tests were significant ( $p < 0.001$ ). For ex vivo experiments, mice were injected with 4T1 cells for tumor extraction and mammosphere plating. Mammospheres exposed to AHCC were significantly different from the control ( $p$ -value  $< 0.001$ ), but not from each other (Fig 2). AHCC was determined non-toxic up to 8mg/ml by MTT assay in MDA-MB-231 cells.

### **3.5.2 Flow cytometry**

Previous studies have shown that BCSCs demonstrate a  $CD44^+/CD24^-$  phenotype with flow cytometry (90). CD44 has been previously been shown to predict the prognosis of TNBC (234). Flow cytometry analysis revealed an average of a 17.47%

decrease of CD44<sup>+</sup>/CD24<sup>-</sup> phenotype cells (representing BCSCs) after 24 hours of exposure to 4mg/ml of AHCC in comparison to the control (Fig 3a-c). This further supports the mammosphere findings, suggesting AHCC targets CSCs in breast cancer.

### **3.5.3 Cell motility**

We exposed attached MDA-MB-231 cells to AHCC over a 72 hour period. An effect on cell motility was seen at 24 hours between the 4mg/ml dose and control ( $p < 0.05$ ), and this difference significantly increased after 72 hours of AHCC exposure ( $p < 0.001$ ) (Fig 4a-b). Samples were additionally compared with the post hoc Tukey's Multiple Comparison Test.

### **3.5.4 MicroRNA profiling and RT2-qPCR**

The miRNA profiling revealed several miRNAs were found to be differentially expressed in MDA-MB-231 mammospheres exposed to AHCC in comparison to the controls. The miRNAs displayed were chosen based on whether they were a factor of 1.5 fold higher or lower in the AHCC group in comparison to the controls (Fig 5). We chose to focus on miR-335 for its documented involvement in many types of cancer, including breast cancer.

To confirm these results, RT2-qPCR analysis of miR-335 revealed an average of 3-fold higher miR-335 expression in MDA-MB-231 mammospheres when exposed to AHCC for 5 hours (Fig 6). Although the profiling was done after a 24 hour AHCC exposure, RT2-qPCR at different time points revealed a more consistent fold change at 5 hours for miR-335.

### **3.5.5 Western blot**

We performed a western blot analysis on MDA-MB-231 cells transfected with a miR-335 mimic or inhibitor. We found a relative increase in TNC protein expression with the addition of the miR-335 inhibitor (Fig 7a-b). This suggests that miR-335 negatively regulated TNC in a TNBC cell line, MDA-MB-231.

### **3.5.6 In vivo study**

A downward trend was observed for tumor mass and volume after mice were gavaged with AHCC, however, no statistically significant difference was observed by the student T test. We also decided to examine the influence of AHCC on miR-335 in an in vivo setting, and found a significantly higher expression in the AHCC group (p-value < 0.01) (Fig 8a-c).

## **3.6 Discussion**

Naturally derived compounds have been shown to be effective in preventing and treating breast cancer. In fact, chemoprevention by targeting Cancer Stem Cells (CSCs) is an important paradigm in therapeutic and adjunct therapy in breast cancer. AHCC has proven to be efficient in reducing tumor growth and metastasis in animal models, particularly when combined with a conventional therapy (43,235,236). Therefore, we aimed to study the underlining mechanisms that drive the anti-carcinogenic effects of AHCC and its relationship to the Cancer Stem Cell Theory in Breast Cancer Stem Cells (BCSCs) (237). Since current therapies do not target CSCs, it is important to conduct research on possible alternative therapies.

Isolation of BCSCs from cancer cell lines demonstrated that AHCC influenced mammosphere growth in a dose dependent manner, not only in MDA-MB-231 cells, but also in MCF-7 and 4T1 cells, suggesting that its effect is not cell type dependent (Fig 1a-d). This was also confirmed by flow cytometry analysis (Fig 3a-c). Although no other studies have examined the effects of AHCC on BCSCs, it has previously been shown to target SOX2 and demonstrated cytotoxicity towards gemcitabine-resistant pancreatic cancer cells (238). We similarly showed an effect on mammospheres cultured from Balb/c mice tumors, confirming that the effects also apply in primary culture (Figure 2). Finally, we observed that AHCC prevents MDA-MB-231 cell migration (Figure 4a-b). Together, these results indicate that AHCC inhibits BCSC growth and inhibits TNBC cell motility. This confirms that AHCC's anti-tumorigenic potential extends to breast cancer and may prevent recurrence by targeting BCSCs. Since BCSCs are known to be epigenetically regulated (239), we decided to explore the effect of AHCC on miRNA profiling.

AHCC was particularly effective on the MDA-MB-231 triple negative cells (with no observed toxicity up to 8mg/ml) (Fig 1a-c). TNBC accounts for 10-20% of diagnosed breast cancers in women globally and has the highest recurrence rate of breast cancer subtypes because it lacks targeted therapies (51,57,62). TNBC is currently treated by radiation, surgery, and chemotherapies like paclitaxel. Paclitaxel is a taxane, which creates aberrant mitotic spindles (240), but this mechanism does not allow for the targeting BCSCs and may even increase their number rather than reduce it (241). When studying the epigenetic mechanisms underscoring the effects of AHCC on the MDA-

MB-231 cells, we found important microRNAs to be involved in the potential inhibitory activity of AHCC against BCSCs. By microRNA profiling, we have shown that several miRNAs, some of which are associated with different clinical-pathological characteristics of breast cancer, such as stemness, invasion and chemoresistance, could be differentially expressed after AHCC administration. For example, miRNAs miR-92a, miR-181a, miR-183\*, miR-335, miR-497, miR-500a, miR-720, and miR-1285 (222,242–248) (Fig 5). The main purpose of this profiling was to choose a target of interest and we elected to further study the particular role of AHCC on tumor suppressor mir-335 for its known involvement in breast cancer metastasis and stem cell maintenance (218,222,249). Confirming miRNA profiling results, RT2-qPCR analysis showed that AHCC significantly upregulated the expression of miR-335 in MDA-MB-231 cell culture (Fig 6).

miR-335 suppresses metastasis and migration via targeting protein pathways, such as that of the progenitor cell transcription factor SOX4 and extracellular matrix component tenascin C (TNC) (218). Several studies have previously linked miR-335 with TNC in cancer, which is also important in breast cancer metastasis and stem cell maintenance (149,156,201). Further, TNC might be involved with the anti-tumoral effects of AHCC through shared immune system targets. TNC inhibits cytotoxic T cell (CTL) activity (169) and AHCC increases it (39), which could influence CSC immune evasiveness and resistance. Therefore, AHCC may increase CTL activity by inhibiting TNC through miR-335 upregulation, and possibly by targeting other miRNAs.

TNC protein expression was found to be increased in the presence of the miR-335 inhibitor, but it was not affected by the miR-335 mimic (Fig 7a-b). One explanation for the ineffective mimic could be timing. In a study examining the effects of miR-335 transfection on hepatic stellate cells, TNC protein expression was not examined by western blot until 6 days after transfection, however, we extracted at 48 hours (224). Nevertheless, other studies have found that a higher miR-335 presence lowers TNC luciferase activity and expression, and inhibits normal breast mammosphere growth (215,218). Thus, both our study and the literature suggest that miR-335 negatively influences TNC and that both miR-335 and its target may play an important role in CSC regulation.

In order to validate the effect of AHCC in an in vivo setting, we used the breast cancer cell model previously established in our lab (250). Although we were not able to see a significant difference in tumor growth with the oral gavage of AHCC, there was a downward trend. One explanation could be due to the dosage used. In this study, the dosage used was 1g/kg/day which was the most common in the literature, however, doses in the literature for mice and rats ranged from 100mg/kg/day to 12.5g/kg/day and typically AHCC was combined with a chemotherapy agent (39,40,43,45,47). Therefore, we cannot establish causation between AHCC and 4T1 tumor growth. However, it is reported in the literature that anti-tumoral mechanisms might target cellular pathways without directly affecting tumoral growth (251). Importantly, there was an increased miR-335 expression in the AHCC mouse tumor samples compared to the controls (Fig 8a-c). This suggests that AHCC also targets miR-335 in vivo. Previous animal studies

with miR-335 have shown that it prevents breast cancer lung and bone metastasis in mice after the injection of MDA-MB-231 into mice (218).

In summary, AHCC is a promising natural product for the complementary treatment of TNBC, previously shown to improve side effects caused by chemotherapy in breast cancer patients (22). This study demonstrated that AHCC has a strong relationship with miR-335 pathways in different biological settings and that it could possibly target the oncogenic protein Tenascin C through miR-335 in a triple negative cell line. Overall, these findings suggest that AHCC effectively targets CSCs, making it an intriguing natural product for further research endeavors.

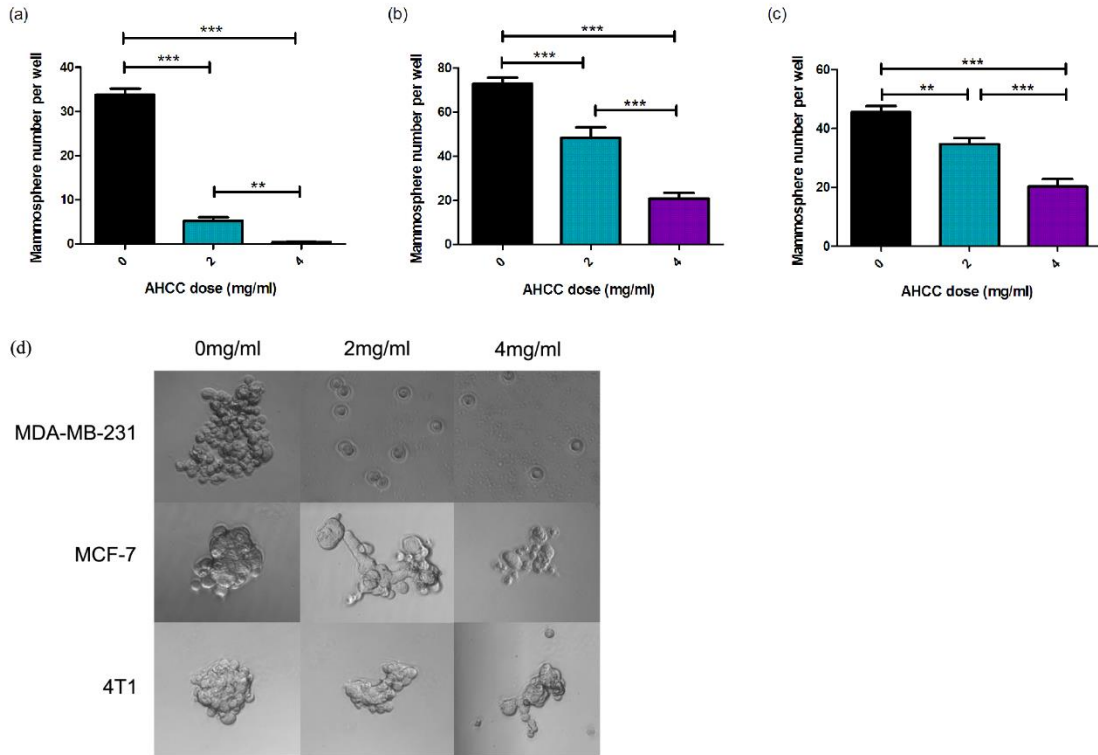
### **Acknowledgements**

This study was financially possible by contributions from the AHCC Research Association and the Canadian Institutes of Health Research (CIHR). We would also like to thank Dr. Hector Hernandez-Vargas for his support and guidance with miRNA analysis.

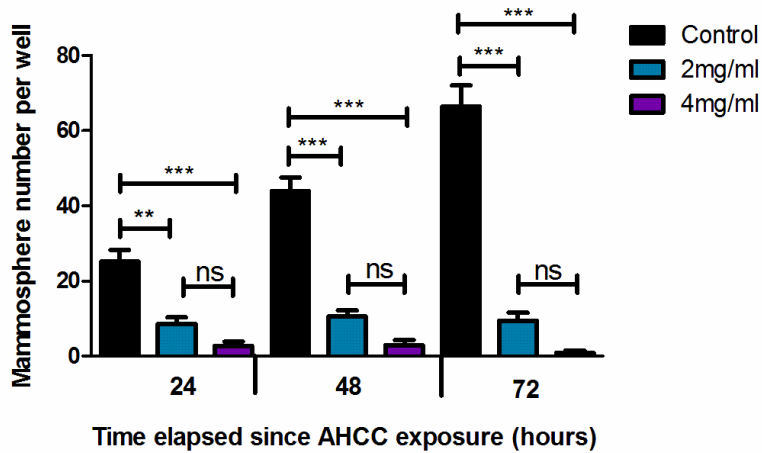
### **Disclosure statement**

The project received financial support from the AHCC Research Association. Dr. Kohei Homma and Dr. Hiroshi Nishioka are employees of Amino Up Chemical Co., Ltd. All other co-authors have no financial interest in the company.

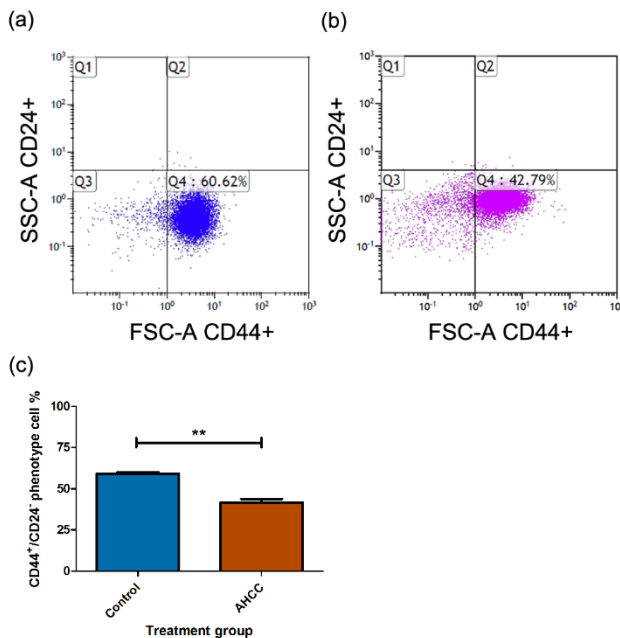
### 3.7 Manuscript figures



**Fig 1a-d. Mammosphere growth after AHCC exposure.** (a) MDA-MB-231 mammosphere growth after 24h exposure to AHCC. Approximately 3600 cells were added per well. Data is a combination of 6 experiments. (b) MCF-7 mammosphere growth after 24h exposure to AHCC. Approximately 5100 cells were added per well. Data is a combination of 3 experiments. (c) 4T1 mammosphere growth after 24h exposure to AHCC. Approximately 1700 cells were added per well. Data is a combination of 3 experiments. (d) Photographs of mammospheres taken with AxioCamMR3 camera on light microscope. Mammospheres were isolated and grown in a 96-well ultra-low attachment plate in DMEM-F12 and spheroid medium at 37°C and 5% CO<sub>2</sub>. All data are presented as mean ± SEM. Significance is represented by \*\* for p < 0.01 and \*\*\* for p < 0.001.

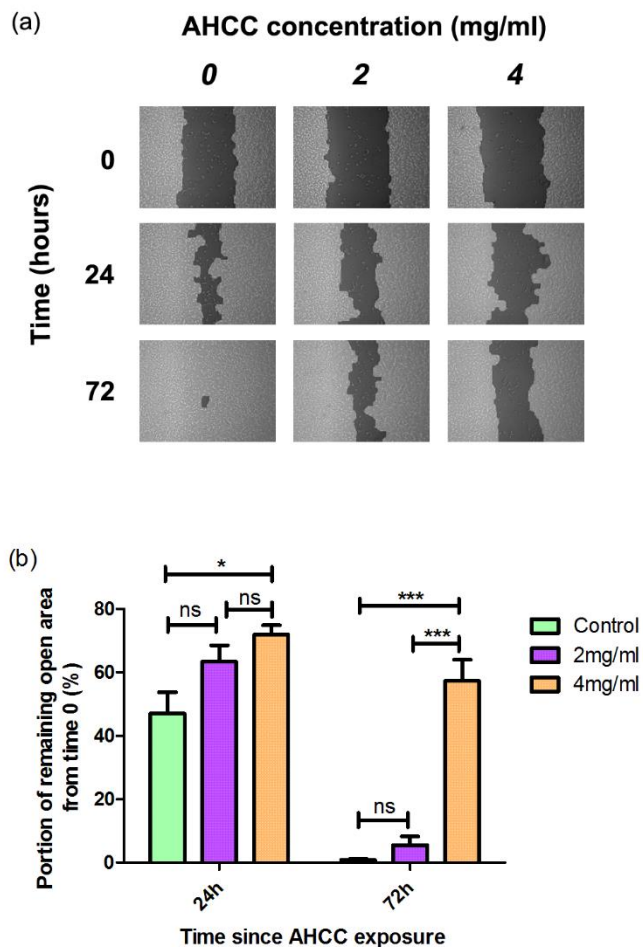


**Fig 2. Ex vivo 4T1 mammosphere growth after 24, 48, and 72 hour exposures to AHCC.** Data are expressed as mean  $\pm$  SEM. Mouse tumors were digested with collagenase and grown in 96-well ultra-low attachment plates at 37°C and 5% CO<sub>2</sub>. Approximately 3400 cells were added per well. Data is a combination of four mice. Significance is represented by \*\*\* for  $p < 0.001$  and non-significance by ns.

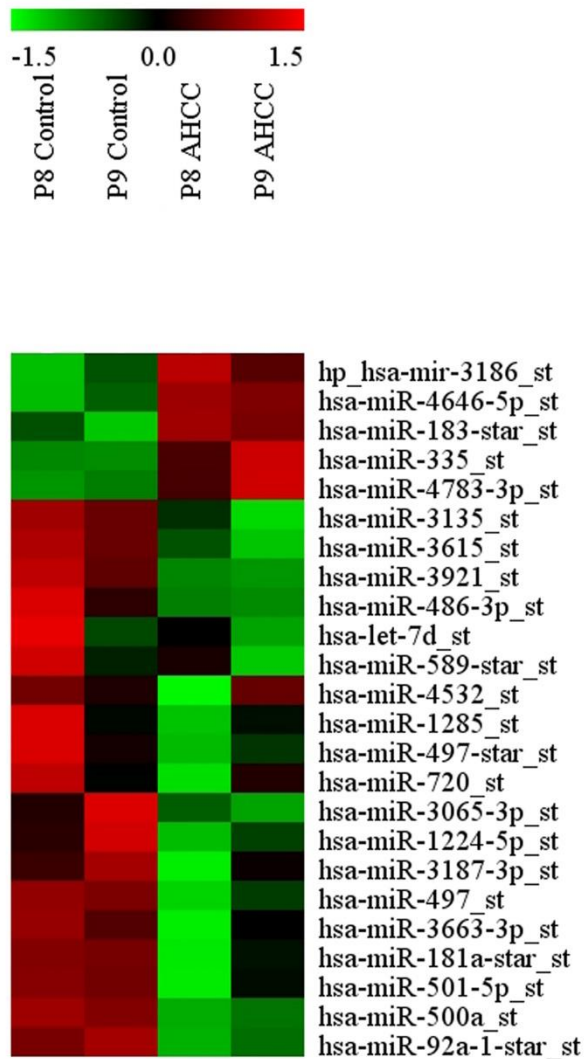


**Fig 3a-c. AHCC reduces CD44<sup>+</sup>/CD24<sup>-</sup> phenotype MDA-MB-231 cells.** (a) Untreated MDA-MB-231 mammospheres. (b) Treated MDA-MB-231 mammospheres. The treatment group was exposed to 4mg/ml of AHCC for 24 hours. (c) Comparison between untreated control and treated AHCC mammospheres with a combination of 3 experiments. Data are presented as mean  $\pm$  SEM. Significance is represented by \*\* for  $p < 0.01$ . MDA-MB-231 mammospheres ( $2 \times 10^5$  cells/ml) were plated in 6-well ultra-low

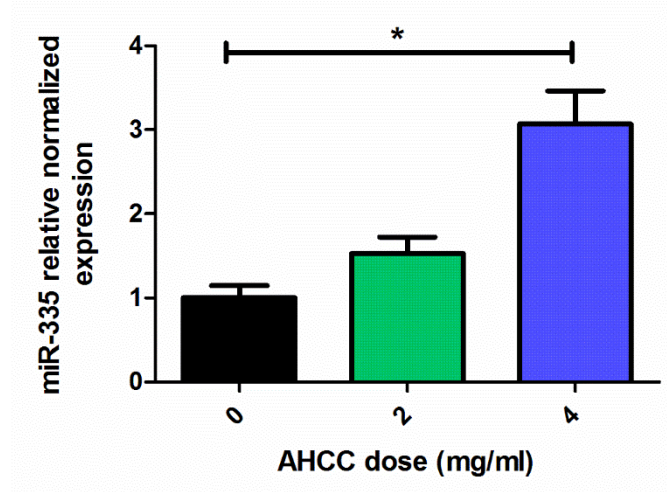
attachment plates in DMEM/F12 and spheroid medium and incubated at 37°C and 5% CO<sub>2</sub>. The cells were exposed to antibodies CD44 and CD24 and profiles were analyzed by flow cytometry.



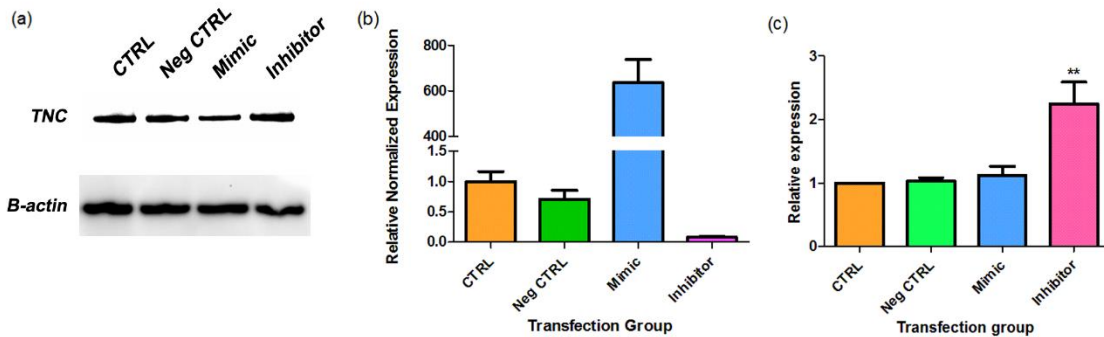
**Fig 4a-b. The influence of AHCC on MDA-MB-231 cell motility.** (a) Photographs from TScratch program for one sample over a period of 72 hours. (b) Combined data from 3 experiments. Data are presented as mean  $\pm$  SEM. Significance is represented by \* for  $p < 0.05$  and \*\*\* for  $p < 0.001$  and non-significance by ns. Cells were grown to 90-100% confluence in a 6 well plate and scratched with a pipette tip down the middle of the well. Photos were taken on a light microscope.



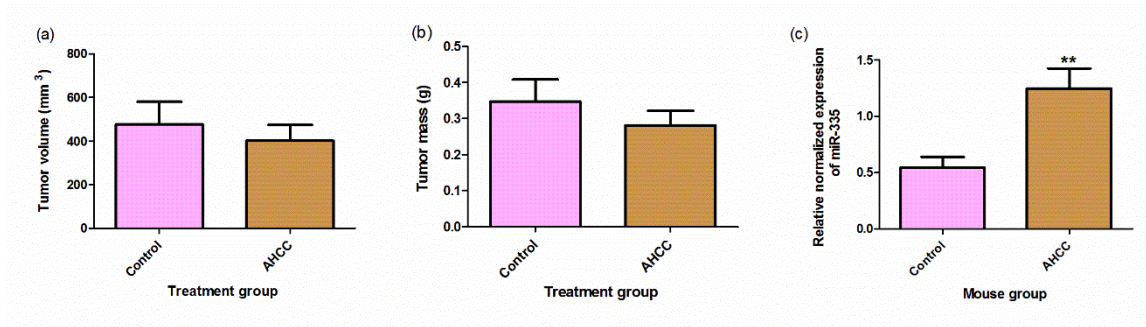
**Fig 5. MicroRNAs differentially expressed in MDA-MB-231 after 24h exposure to AHCC.** The microRNAs were chosen based on whether their fold change was  $>$  or  $<$  by a factor of 1.5 in the AHCC group in comparison to the control. The profiling heat map represents a calculated z-score (-1.5 to 1.5 from mean). Each column and row represents a sample and specific miRNA expression, respectively.



**Fig 6. AHCC upregulates miR-335 expression in MDA-MB-231 mammospheres.** Results are a combination of means  $\pm$  SEM from 3 separate experiments. MDA-MB-231 cells were plated in 6-well ultra-low attachment plates in DMEM-F12 and spheroid medium and incubated at 5% CO<sub>2</sub> and 37°C. Cells were exposed to varying concentrations of AHCC for 5 hours. MiR-335 expression levels were measured by RT2-qPCR in comparison to reference snRNA U6. Significance is represented by \* for  $p < 0.05$ .



**Fig 7a-c. miR-335 inhibitor elevates TNC expression in MDA-MB-231 cells.** (a) Sample western blot photographs for Tenascin C (TNC) and control beta-actin (B-actin) as shown on Image Lab. (b) Combined western blot data from Image Lab calculations. Data are expressed as means  $\pm$  SEM and are a combination of 3 experiments. MDA-MB-231 were transfected with a negative control, mimic, or inhibitor. Samples were analyzed by western blot. Significance is represented by \*\* for  $p$ -value  $< 0.01$ .



**Fig 8a-c. Mouse tumor measurements and miR-335 expression.** (a) Mouse inner tumor volume. (b) Mouse tumor mass. Data are presented as mean  $\pm$  SEM. Tumor volume was calculated by the equation  $0.5 \times d^2 \times D$  where  $d$  is the smaller measured diameter and  $D$  is the larger measured diameter. Mice were gavaged for 2 weeks, injected with 4T1 cancer cells, and gavaged again for 18-23 days following injection. Tumors were extracted from mice following gavage period. The means were calculated with eleven treatment mice and nine control mice. (c) Change in miR-335 expression in mice gavaged with AHCC. Mice were gavaged with 1g/kg/day AHCC in 1% sucrose water or with 1% sucrose water (control). After 2 weeks of gavage, mice were injected with 4T1 cells and sacrificed 18-21 days following. Tumors were minced and digested for RT2-qPCR analysis. Data are presented as mean  $\pm$  SEM. These are the results of two RT2-qPCR runs with a total of 8 mice in the control group and 9 mice in the AHCC group. Quartile outliers were removed and \*\* indicates a significance of p-value  $< 0.01$ .

## **Chapter 4 : General discussion**

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Natural products such as AHCC are commonly used as forms of CAM by breast cancer patients worldwide. Although many studies support the use of AHCC for immune health and as a complementary medicine to chemotherapy treatments, little is known about its targets and pathways in cancer. To our knowledge, this study is the first to examine the effects of AHCC on Cancer Stem Cells, microRNA and protein targets in breast cancer. Our objectives were to study if and how AHCC influenced TNBC mammosphere growth and invasiveness in in vitro, ex vivo, and in vivo settings.

## **The influence of AHCC on breast cancer stem cells and cancer cell invasion**

Our first aim was to confirm whether AHCC influenced breast cancer stem cell (BCSC) growth and TNBC metastatic potential. Cancer Stem Cells (CSCs) are now considered an important paradigm in cancer prevention and adjunct therapy (93). We used a mammosphere model to study BCSCs and demonstrated that AHCC influences mammosphere growth in a dose dependent manner in vitro, not only in MDA-MB-231 cells, but also MCF-7 and 4T1 cells to a lesser extent, suggesting that its effect is not cell type dependent (Fig 1a-d). The stronger effects seen on MDA-MB-231 cells could potentially be due to their different morphology, since MCF-7 and 4T1 cells have a round shape whereas MDA-MB231 cells have a grape cluster shape (Fig 1a-d). Since the mammosphere model does not perfectly isolate CSCs, we also performed flow cytometry analyses on MDA-MB-231 cells and confirmed AHCC influence on the CSC population by looking at the CD44 and CD24 markers (Fig 3a-c), since BCSCs have previously been shown to exhibit a CD44<sup>+</sup>/CD24<sup>-</sup> phenotype (90). Furthermore, we observed a significant influence of AHCC on MDA-MB-231 cell migration by measuring cell motility (Fig 4a-b). This suggests that AHCC may be able to inhibit TNBC metastatic properties. Together, these results indicate that AHCC inhibits BCSC growth and TNBC migration in an in vitro setting. This has important implications for AHCC in cancer resistance prevention.

## **The influence of AHCC on breast cancer stem cells in ex vivo and in vivo settings**

Our second initiative was to validate the in vitro anti-tumoral effects of AHCC in primary CSC culture and in an animal model. Accordingly, AHCC significantly inhibits

mammosphere growth in ex vivo culture from tumors extracted from Balb/c mice injected with 4T1 cells (Fig 2). This demonstrates that AHCC also influences more natural, unaltered BCSCs, eliminating the concerns of mutation that follow passaged cell culture.

We also wanted to examine the anti-tumoral capabilities of AHCC in vivo. The effective dosage will depend on metabolism and AHCC has not been tested in vivo for breast cancer without the addition of a chemotherapy agent (236). Since it is already known to improve the effects of chemotherapy agents, this study aimed to further analyze its chemopreventive effects in a non-adjunct therapy setting. The mean tumor size of the AHCC group was smaller than the control, but not significantly. The lack of influence of AHCC on in vivo tumor growth could be due to not using a high enough dosage, since the doses in the literature for mice and rats ranged from 100mg/kg/day to 12.5g/kg/day (39,40,43,45,47), and/or to the low number of mice used in each group. We chose a dose of 1g/kg/day since it was the most commonly used, however, it may not have been high enough for using AHCC without the addition of a chemotherapy agent. Further, it could be that AHCC influences other aspects of breast cancer than tumor size that we did not measure, such as metastasis and survival. Other studies have shown that products may influence survival while not affecting tumor size (247). It could also simply mean that AHCC is not potent enough on its own in an in vivo setting and instead works better as a complementary therapy. Further studies could examine earlier time points, use higher doses or less stressful administration methods than gavage, and raise the number of mice in each group. Another point to note is that AHCC was less effective on the 4T1 cell line in comparison to the MDA-MB-231 cell line, which could be why

there is a lack of effect in this in vivo experiment. Overall, these results indicate that AHCC's influence extends to an ex vivo setting and herein supports its confirmed effects in in vitro cell lines.

### **AHCC targets miRNAs in triple negative breast cancer stem cells in vitro and in vivo**

Cancer Stem Cells are known to be epigenetically modulated. The study of the miRNAome by microarray profiling proved that there are many miRNAs involved in many characteristics of breast cancer aggressiveness, such as stemness and chemoresistance, that were differentially expressed (Fig 5). AHCC influenced the expression of miR-92a, miR-181a, miR-183\*, miR-335, miR-497, miR-500a, miR-720, and miR-1285, all well-known miRNAs for their involvement in breast cancer development (222,242–248). It should be noted that a small sample size was used, limiting the power of the data, however, the purpose was to find a target of interest to further pursue by RT2-qPCR analysis. Since miR-335 had the largest fold change of these and was one of the most relevant in the literature, we decided to pursue further validation studies on miR-335. AHCC upregulated miR-335 expression by about 2-fold in the profiling and by about 3-fold in RT2-qPCR, likely because of the different time points it was analyzed at; 24 and 5 hours, respectively (Fig 6). miR-335 is well known to be important for breast cancer metastasis and for stem cell maintenance (202,218,222). This makes it a very intriguing target for AHCC in BCSCs.

To examine whether AHCC also influenced miR-335 in an in vivo setting, we performed RT2-qPCR analyses on RNA extracted from mouse tumors. We examined

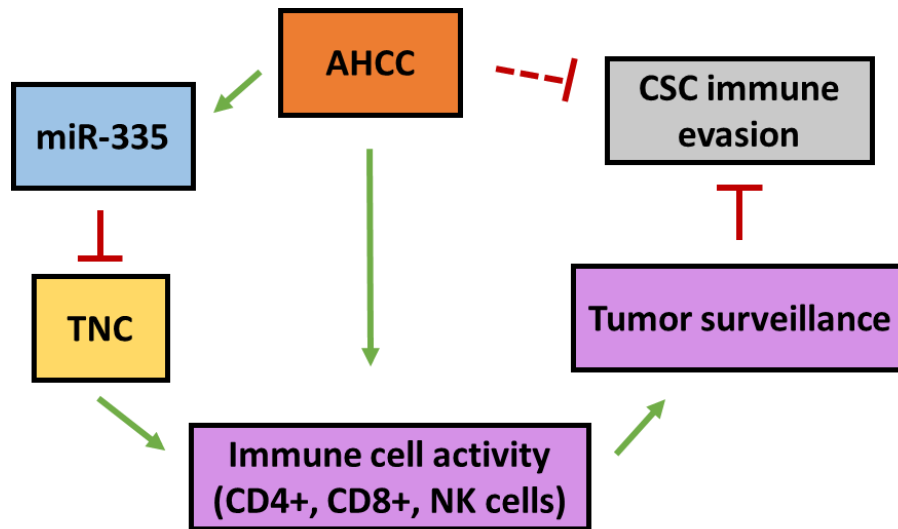
three tumor suppressor miRNAs, miR-145, miR-335, and miR-365 in the non-gavaged mouse group. We used miR-145 and miR-365 as comparisons, since they are also known to be involved in breast cancer pathways (4,211). Although the expression varied between mice tumor samples, there appeared to be a trend in which if one miRNA was highly expressed in a mouse tumor sample, the other miRNA were also more likely to be highly expressed in that same mouse sample (Figure A.11, Appendix 1). This could indicate that they are involved in related pathways in cancer management. Furthermore, we ran a RT2-qPCR with the gavaged mouse tumor samples, revealing a significantly higher expression of miR-335 in the group that received AHCC (Fig 8a-c). This is an interesting significant result that further supports the other findings in this study and suggests that AHCC additionally targets miR-335 in an in vivo setting. We further expanded our analysis to study if there was a trend between tumor growth and miR-335 expression in the non-gavaged mice and found a negative correlation ( $R^2 = 0.8344$ ) for the five mice run on the same RT2-qPCR (Figure A.12, Appendix 1). This was significant ( $p\text{-value} < 0.05$ ) and directionally supports the literature, since miR-335 is expected to be downregulated in breast cancer (213). We additionally compared miR-335 expression in the gavaged mice to their tumor volumes, and unlike the non-gavaged mice, miR-335 expression did not significantly correlate with tumor volume (Figure A.13, Appendix 1). This suggests that the stress caused by gavage may have influenced tumor growth. However, it should still be kept in mind that the lack of significance could indicate that miR-335 does not influence 4T1 tumor growth in mice.

We also examined mammosphere growth and cell motility of miR-335 transfected cells, but did not see any significant change with the addition of the mimic or

inhibitor (Figure A.5). This differs from previous findings, which showed that transfection with a miR-335 mimic prevents MDA-MB-231 cell migration (217). Therefore, we cannot confirm that miR-335 alone is responsible for the mammosphere reduction and slower cell migration seen in AHCC treated cells. It is likely that AHCC targets several miRNAs that work synergistically (such as those seen in profiling) and possibly other targets in the immune system and inflammation pathways. For instance, a previous study showed that the concurrent knock-down of miR-335 with miR-21 and miR-153 significantly increased the expression of the Notch signaling ligand JAG1, whereas the increase in expression was insignificant when these miRNAs were knocked down alone (221). This indicates that miR-335 may need to work with other miRNAs to produce its anti-CSC effects.

Additionally, miR-335 has previously been linked to Tenascin C (TNC), which is also important in breast cancer metastasis and stem cell maintenance (162,163). The literature observed that miR-335 upregulation inhibits TNC protein expression in hepatic stellate cells (224). Our study determined that inhibition of miR-335 upregulated TNC protein expression, however, we could not confirm that upregulation of miR-335 down-regulated TNC protein expression (Fig 7a-c). In the study examining the effects of miR-335 transfection on hepatic stellate cells, TNC protein expression was not examined by western blotting until 6 days after transfection and we collected our protein after 48 hours (224). Although this is a different cell line than the one used in the previous study, it could mean that we examined the effects too early and missed the time window when miR-335 fully impacts protein expression.

What mechanisms of action can be elucidated from these findings that may be clinically important? AHCC has been reported to exert immunoprotective effects in many illnesses such as bacterial and viral infections and cancer (22,40,41,43). Precisely, it enhances CD4<sup>+</sup> and CD8<sup>+</sup> T cell proliferation and increases NK cell activity (38,39,42). An in vivo study in mice found that orally administered AHCC increased the number of tumor Ag-specific IFN- $\gamma$  producing CD8<sup>+</sup> T cells (38). Therefore, it may have a mechanism by which it prevents CSC immune evasion, possibly by limiting TNC expression and thus allowing T cell maturation and proliferation (Figure 4.1). Since TNC is influenced by miR-335, AHCC may indirectly lead to TNC reduction through the elevation of miR-335 expression. However, this pathway involving the immune system does not entirely explain why AHCC influenced mammosphere growth in vitro since there is no immune system present. It is possible that AHCC influences multiple pathways, perhaps by also targeting other miRNAs or by targeting transcription factors in the EMT pathway. Also, miR-335 and TNC may influence CSC growth through other mechanisms. For instance, another study found that miR-335 suppressed invasion by inhibiting SOX4 and TNC in vitro, measured by a trans-well invasion assay with LM2 cells (lung cancer) (218). No other studies have yet examined AHCC's mechanisms of action in BCSCs and we have just scratched the surface of possibilities for how it works.



**Figure 4.1. Theory for the prevention of CSC immune evasion by AHCC.** AHCC may prevent CSC immune evasion partly by targeting miR-335 pathways, since TNC directly targets immune cells like cytotoxic T cells (CD8+) and AHCC is also known to target these, although its exact mechanisms of action are unknown. If AHCC were to inhibit TNC action indirectly through miR-335, it would allow for its previous effects seen on increased immune cell activity and increased tumor surveillance (38,39,42,169).

## Conclusion

CAM is used by more than half of breast cancer patients and, therefore, should be studied in greater detail to determine its effectiveness and possible side effects when used with current cancer therapies. AHCC is a promising form of CAM used worldwide for immunoprotection and as adjunct therapy for cancer. It promises to be a good complementary medicine as it has been shown to reduce chemotherapy side effects such as nausea, while also helping fight tumor growth. Our study demonstrated that it prevents BCSC growth and migration in vitro, possibly by targeting miRNAs such as miR-335 and proteins like TNC.

## **Future directions**

This study and the literature have proven AHCC to be an intriguing natural product for the treatment of cancer. In particular, it has been shown to be a good complementary medicine to conventional therapies, not only improving chemotherapy symptoms such as nausea, but reducing tumor growth in animal studies. In this study, we have further elucidated its role on Cancer Stem Cells and supported its potential use in cancer therapies and chemoprevention. Since we were not able to confirm AHCC's effect on tumor growth in vivo, further studies should examine it perhaps at a different time point or at a higher dose. They could also pair it with a positive control, such as paclitaxel typically used to treat TNBC. Also, additional studies should further examine AHCC's role on CSCs derived from different types of cancer, more specifically from hepatocarcinoma, since the effectiveness of AHCC has been proven in clinical studies with liver patients. Furthermore, it would be interesting to examine the effect of AHCC on ex vivo CSC growth from cancer patients and relate its anti-tumoral effects to different stages and types of cancer. Additionally, since miR-335 and TNC appear to be important players in CSC pathways and breast cancer, these should be examined in further detail. For instance, TNC could also be targeted by TNC-specific antibodies as an immunotherapy. Overall, our study contributed to building evidence-based data regarding the usage of this natural product in cancer.

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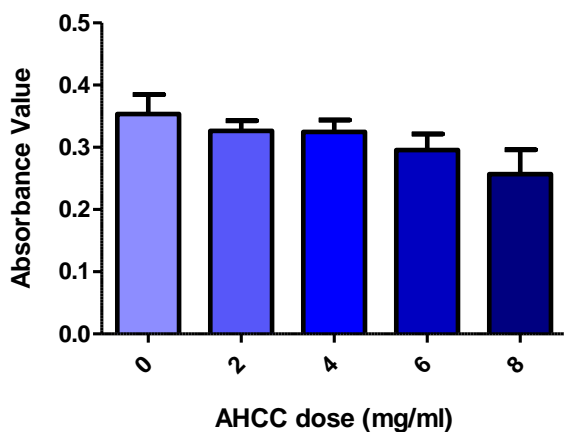
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## Appendix A : Unused data

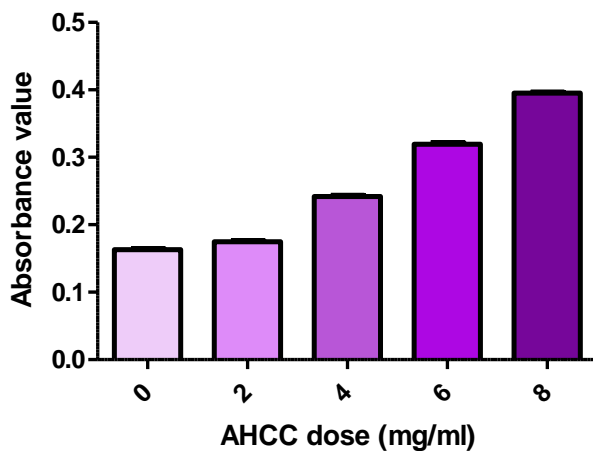
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### Cell viability

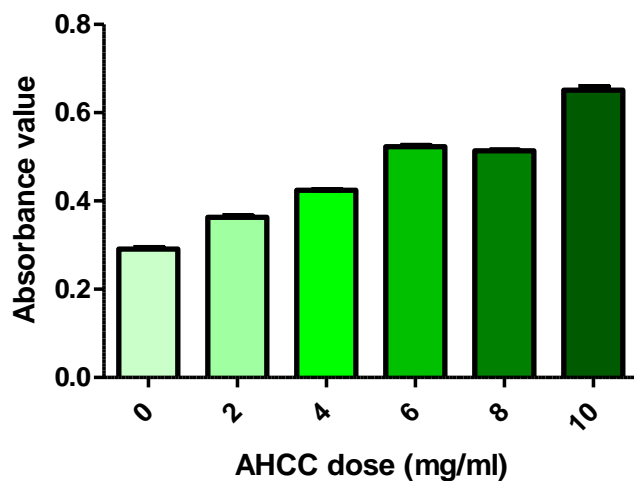
Originally, we tried to measure cell viability and proliferation of MDA-MB-231 cells with LDH and WST-1 tests. We began with these rather than MTT because it was possible to measure the cell viability of suspended mammospheres (Figure A.1). In the LDH test, cell death increases with the color as dead cells release LDH into the media. On the other hand, the WST-1 test measures proliferation and should have a higher absorbance value with more viable cells in the wells. Since both tests showed an increase in absorbance with concentration (Figures A.2 and A.3), we decided to measure the AHCC absorption in media alone at a wavelength within the range of these tests, 450nm. We discovered that the AHCC would indeed influence the results of these tests due to its yellow-brown color, as it increased in absorbance despite no cells being present (Figure A.4).



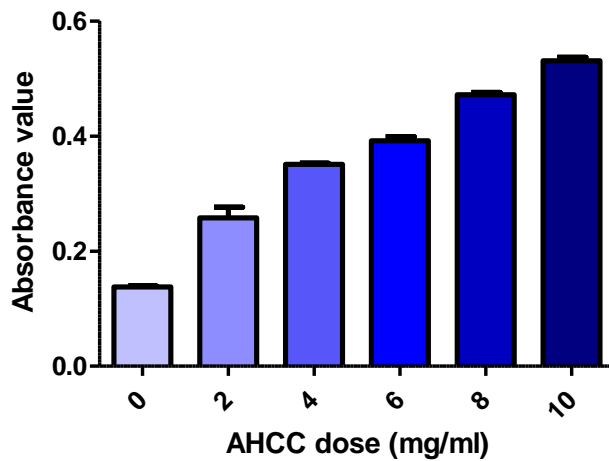
**Figure A.1. AHCC does not affect MDA-MB-231 cell viability.** An MTT test was performed on MDA-MB-231 cells exposed to AHCC for 24 hours. Cells were cultured in RPMI and incubated at 37°C and 5% CO<sub>2</sub>. The highest dose examined was 8mg/ml. Data are presented as mean ± SEM.



**Figure A.2. LDH test on MDA-MB-231 cells exposed to AHCC.** An increase in absorbance from this test would normally indicate that AHCC is causing cell death with an increasing concentration. Absorbances were measured with a u-Quant spectrophotometer.



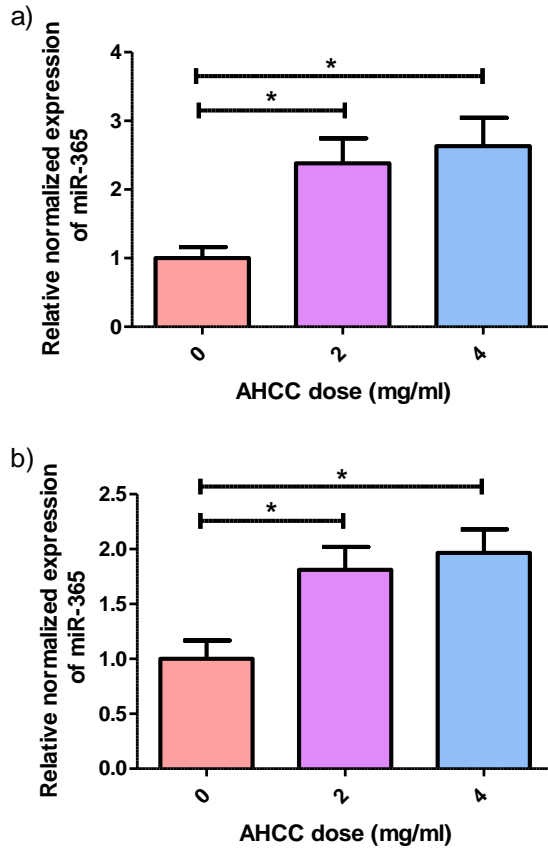
**Figure A.3. WST-1 test of MDA-MB-231 cells exposed to AHCC.** An increase in absorbance for this test would normally indicate that AHCC is increasing the proliferation of viable MDA-MB-231 cells. Absorbances were measured with a u-Quant spectrophotometer.



**Figure A.4. The absorption of AHCC plated in media without cells.** AHCC was plated in DMEM-F12 medium at varying concentrations and analyzed with a u-Quant spectrophotometer.

## RT2-qPCR

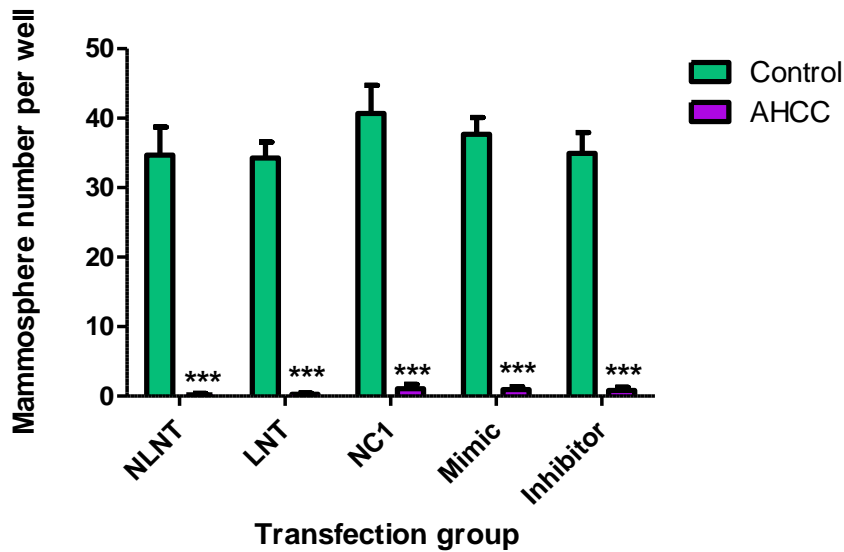
When performing RT2-qPCR with miR-335, we used miR-365 as a comparison to make sure the results seen were not due to an overall elevation of miRNA in the cells. We found that miR-365 did not get influence by AHCC very much in MDA-MB-231 mammospheres, however, it was elevated in regular MDA-MB-231 cells. It was slightly more elevated after 5 hours of exposure to AHCC compared to 24 hours of exposure (Figure A.5a-b).



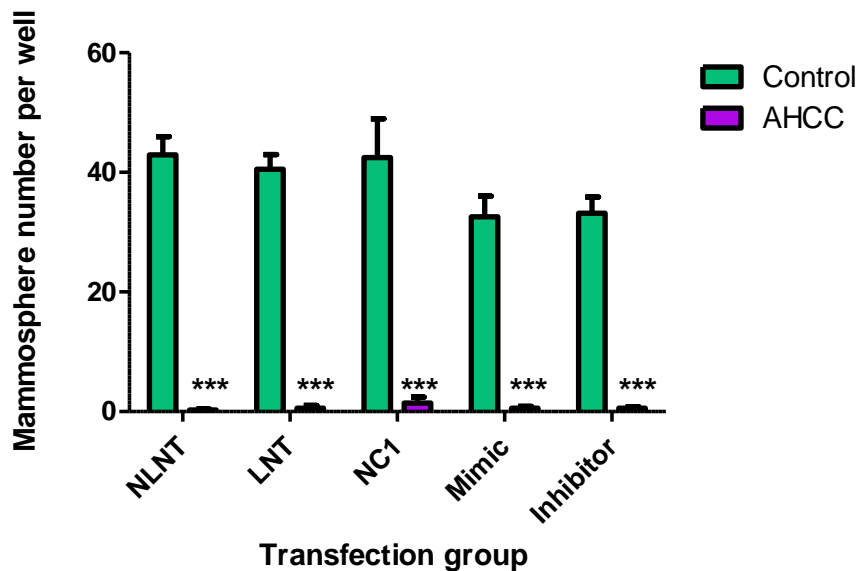
**Figure A.5a-b. AHCC upregulated the expression of miR-365 in MDA-MB-231 cells. (a) 5 hours exposure and (b) 24 hours exposure.** Cells were plated in RPMI medium in a 6-well plate and incubated at 5% CO<sub>2</sub> and 37°C.

## Transfection

We decided to see what would happen if we added AHCC to transfected cells. We were curious as to whether the potency of AHCC could overcome the inhibitor and surpass the mimic. Mammosphere results demonstrated the same significant effect by 4mg/ml of AHCC on all transfection groups, but control groups did not significantly differ from each other in MDA-MB-231 cells or 4T1 cells (Figures A.6 and A.7). RT2-qPCR results demonstrated a higher expression of miR-335 with the addition of AHCC in most groups in both MDA-MB-231 and 4T1 cells (Figures A.8 and A.9).

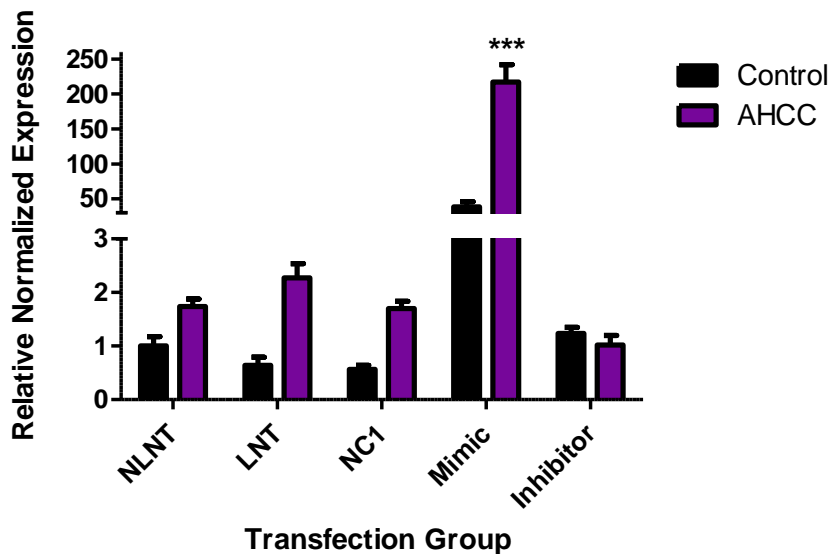


**Figure A.6. AHCC reduced mammosphere growth in transfected MDA-MB-231 cells.** Mammospheres were isolated and grown in a 96-well ultra-low attachment plate in DMEM-F12 and spheroid medium at 37°C and 5% CO<sub>2</sub>. Data are a combination of 4 experiments. All data are presented as mean ± SEM. Significance is represented as \*\*\* for p < 0.001. NLNT= no lipofectamine no template, LNT = lipofectamine no template, NC1 = negative control 1.

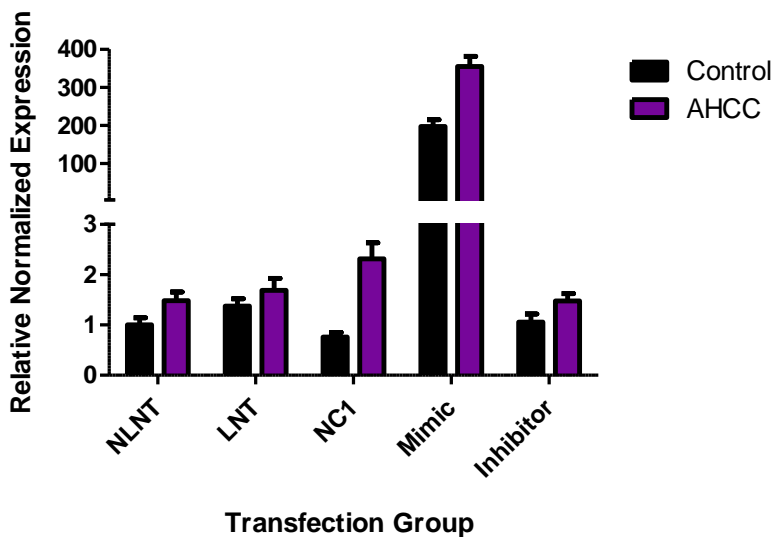


**Figure A.7. AHCC reduced mammosphere growth in transfected 4T1 cells.** Mammospheres were isolated and grown in a 96-well ultra-low attachment plate in DMEM-F12 and spheroid medium at 37°C and 5% CO<sub>2</sub>. Data is a combination of 3 experiments. All data are presented as mean ± SEM. Significance is represented as \*\*\*

for  $p < 0.001$ . NLNT= no lipofectamine no template, LNT = lipofectamine no template, NC1 = negative control 1.



**Figure A.8. AHCC elevated miR-335 expression in transfected MDA-MB-231 cells.** Cells were isolated and grown in a 6-well plate in RPMI medium at 37°C and 5% CO<sub>2</sub> and exposed to AHCC for 5 hours. Data is a combination of 3 experiments. All data are presented as mean ± SEM. Significance is represented as \*\*\* for  $p < 0.001$ . NLNT= no lipofectamine no template, LNT = lipofectamine no template, NC1 = negative control 1.

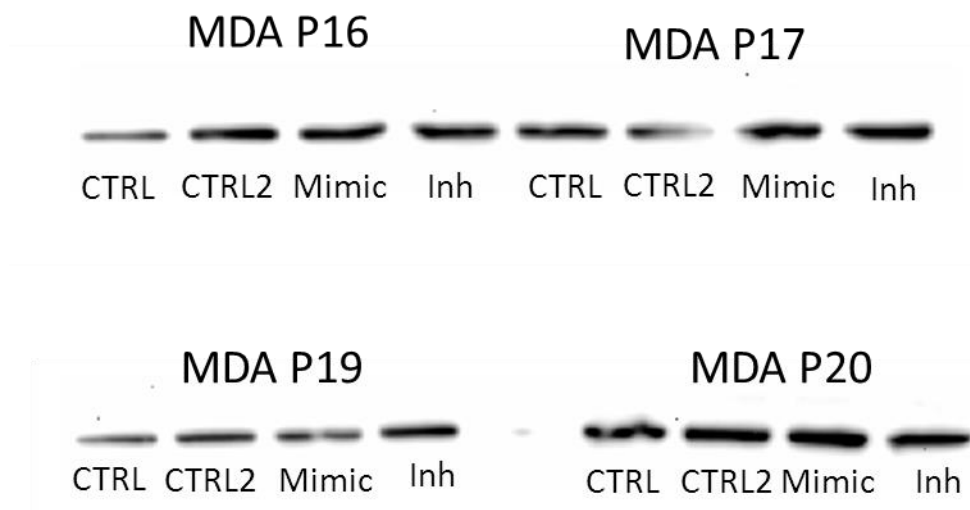


**Figure A.9. AHCC did not significantly effect miR-335 expression in transfected 4T1 cells.** Cells were isolated and grown in a 6-well plate in RPMI medium at 37°C and 5% CO<sub>2</sub> and exposed to AHCC for 5 hours. Data is a combination of 3 experiments. All

data are presented as mean  $\pm$  SEM. NLNT= no lipofectamine no template, LNT = lipofectamine no template, NC1 = negative control 1.

## Western blot

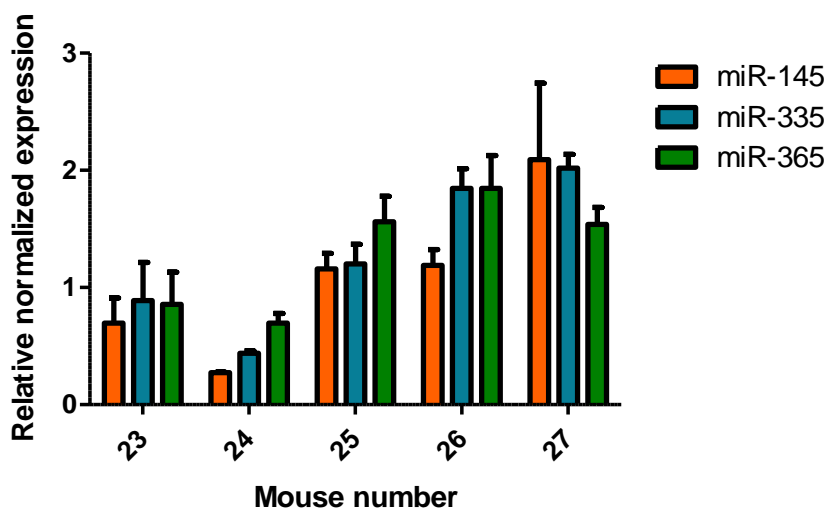
Besides working with TNC in western blot, we also tried examining Sox4. However, the first antibody we used from Abcam was non-specific and showed bands all across the membrane. The second antibody we tried from Santa Cruz Biotechnology had one solid band (Figure A.10) for all samples, but at the incorrect location on the membrane. Due to lack of time, we decided to then focus on Tenascin C, whose antibody did work.



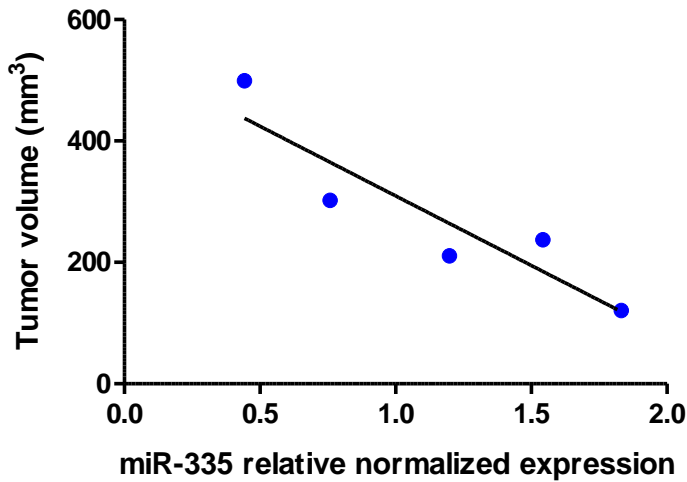
**Figure A.10. Sox 4 expression in miR-335 transfected MDA-MB-231 cells.** Protein was extracted from cells 48 hours after transfection. Sox 4 antibody from Santa Cruz Biotechnology was used. CTRL indicates no lipofectamine and no target, CTRL2 indicates lipofectamine and a nonsense target, Mimic indicates the miR-335 mimic, and Inh indicates the miR-335 inhibitor. Bands were not located at the correct location. CTRL = no lipofectamine no template, CTRL2 = negative control.

## In vivo

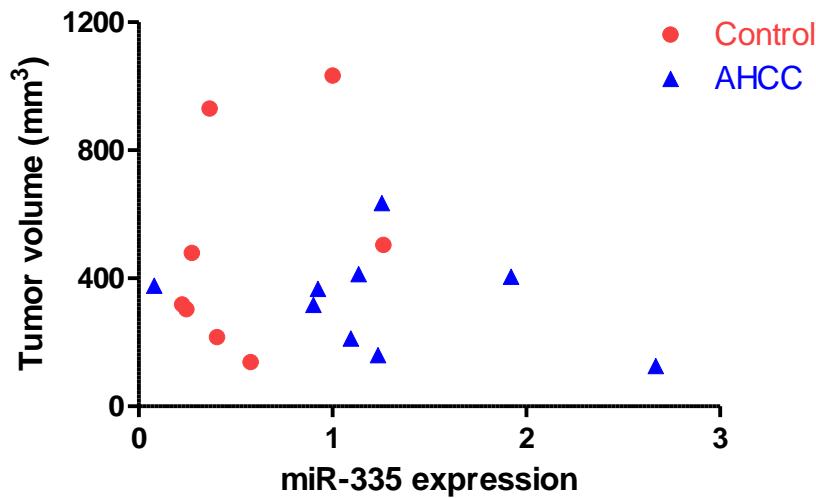
There were several measurements taken for the mice that were not included in the publication due to lack of significance. Additionally, we did a few RT2-qPCR runs with digested mouse tumor samples in the third experiment. In Figure A.11, there appears to be a trend for 3 miRNAs examined, miR-145, miR-335, and miR-365 in the tumor tissues of the non-gavaged mice prepared for ex vivo mammosphere studies. All of these miRNAs are typically downregulated in breast cancer (252–254). We further decided to look at the relationship between their miR-335 expression and tumor volume. There was a significant correlational relationship of  $R^2 = 0.8344$  (p-value < 0.05) in the non-gavaged mice (Figure A.12). However, this was one RT2-qPCR run and the same pattern was not observed in the gavaged mice (Figure A.13), we opted not to include this data in the paper.



**Figure A.11. MicroRNA expression of non-gavaged mouse tumor tissues.** Mice were injected with 4T1 cells and sacrificed 18-21 days following. Tumors were minced and digested for RT2-qPCR analysis. Data are presented as mean  $\pm$  SEM. These are the results of one RT2-qPCR run. Data is normalized to snRNA U6 expression.



**Figure A.12. Correlation between miR-335 expression and tumor volume in non-gavaged mice.** Mice were injected with 4T1 cells and sacrificed 18-21 days following. Tumors were minced and digested for RT2-qPCR analysis. Tumor volume was calculated by the equation  $0.5xd^2xD$  where d is the smaller measured diameter and D is the larger measured diameter. 5 tumor samples were included in this analysis.  $R^2 = 0.8344$  and p-value  $< 0.05$ .



**Figure A.13. Correlation between miR-335 expression and tumor volume in gavaged mice.** Mice were injected with 4T1 cells and sacrificed 18-21 days following. Tumors were minced and digested for RT2-qPCR analysis. Tumor volume was calculated by the equation  $0.5xd^2xD$  where d is the smaller measured diameter and D is the larger measured diameter. 8 control and 9 AHCC mice tumor samples were included in this analysis. Control  $R^2 = 0.1311$  and AHCC  $R^2 = 0.1041$  (p-value  $> 0.05$ ).

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