Chapter 11

Plant Based Natural Products and Breast Cancer: Considering Multi-Faceted Disease Aspects, Past Successes, and Promising Future Interventions

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Additional information is available at the end of the chapter

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1. Introduction

1.1. Dealing with the burden

World-wide, approximately 410,000 of the estimated 1 million females diagnosed annually with breast cancer will die from the disease [1]. According to Canadian Cancer Statistics, breast cancer continues to be the most commonly diagnosed cancer among Canadian women over the age of 20 (excluding non-melanoma skin cancer) and is the second leading cause of cancer deaths [2]. In 2012, it was estimated that 22,700 Canadian women would be diagnosed with breast cancer and 5,200 would succumb to the disease [2]. Furthermore, the recently published Prince Edward Island (PEI) Cancer Trends Report outlining cancer cases diagnosed from 1980 to 2009 indicates that in PEI, breast cancer is the most commonly diagnosed cancer in women [3]. While the overall incidence rate of breast cancer has increased marginally (0.5% per annum) and mortality rates have stabilized since 1992, the long-term survival in PEI women is significantly lower than the Canadian rate [3]. In summary, breast cancer continues to be a world-wide issue requiring attention. Increased awareness and advanced screening, resulting in early detection, is attributed to the increased incidence of the disease and such factors have led to a high percentage (approximately 95%) of all breast cancer patients being initially diagnosed with curable disease [4]. However, 30-40% of patients diagnosed with curable breast cancer succumb to disease reoccurrence [5]. Although increased diagnosis and advances in the treatment of breast cancer are clearly contributing factors in disease progression, eradication of residual malignancies and metastatic tumors via a systemic approach is considered the key

for success in treating cancer and increasing cancer patient survival [6]. There is an urgent need to explore agents that will be effective in preventing and treating metastasis of breast tumors.

1.2. The biology of breast cancer

In general, cancer occurs when a normal cell accumulates genetic and/or epigenetic changes caused by the activation or amplification of oncogenes and/or the mutation or loss of tumor suppressor function, resulting in the ability to proliferate indefinitely [7]. While these specific alterations lead to the transformation of the normal cell and partly determine the characterization of the tumor, the cell of origin and tumor (micro-) environment are also considered important factors contributing to tumor cell establishment, progression and therapeutic resistance [8].

2. Clinical characteristics and classification of breast cancer

2.1. Classifying breast cancer

Breast cancer classifications are largely explained by differences in tumor characteristics as determined by tumor appearance, histology, tumor marker expression (immunohistochemistry) or receptor status, and gene expression profiles. Alone, or in combination, these aspects can influence treatment, response and prognosis.

2.1.1. Histology

Breast cancer represents many different histologies, however the majority (estimated to be more than 85%) of breast cancers are collectively derived from the epithelium lining in the ducts or lobes, and are classified as mammary ductal or lobular carcinoma [9]. Furthermore, this classification can be defined as *in situ* (meaning the proliferation of cancer cells within the epithelial tissue without invasion of the surrounding tissue) or *invasive* whereby the surrounding tissue is affected [9]. Included in the histological description of breast cancer is the status of invasion to the perineural and/or lymphovascular space and the presence of such is associated with more aggressive disease [10].

2.1.2. Grade

Tumor grade is determined by comparing differentiation in normal and cancerous breast tissues. Normal cells in the breast become differentiated and acquire specific shapes and forms that reflect their function as part of the mammary system [9]. When cell division becomes uncontrolled, differentiation is lost. Breast cancers are either well differentiated (low grade), moderately differentiated (intermediate grade), or poorly differentiated (high grade) [9]. This progressive loss of features seen in normal breast cells is indicative of disease progression and poorly differentiated or high grade cancers have a worse prognosis than others [11].

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2.1.3. Stage

Between 1943 and 1952, Pierre Denoix devised the TNM staging systems for all solid tumors to classify the progression of cancer [12]; the acronym can be explained by the fact that this model utilizes the size and extension of the primary tumor (T), its spread to the lymph nodes (N), and the presence of metastases (M). Although it is not utilized for all cancers (i.e.: brain and spinal cord cancer), the TNM system has progressed to the 7th textbook edition (TNM Classification of Malignant Tumors) [12] and remains the major system by which breast cancer is staged. An increase in stage number is based on larger tumor size, nodal spread (in particular, the sentinel node*) and metastasis and is positively correlated with a worse prognosis [13, 14].

* Certain cancers spread in a predictable manner, from the site of origin to nearby lymph nodes (lymph glands) and then to other parts of the body. The very first draining lymph node is termed the "sentinel node" and is important in the sentinel lymph node biopsy (SLNB) technique utilized to stage the spread of certain types of cancer. The absence of cancer within the sentinel lymph node indicates that there is a high likelihood that the cancer has not spread to any other area of the body. Lymph node metastasis is considered one of the most important predictive signs in breast cancer, and thus can serve to guide the surgeon/oncologist to the appropriate therapy.

2.2. Receptor status and gene expression profiles

Recent progress in molecular technologies has led to distinct breast cancer categories and five distinct tumor types and a normal breast-like group have been identified to date based on gene expression profiling [15], as summarized in Table 1.

Molecular Sub-type based on Gene Expression Profiling	Receptor Status based on Immunohistochemistry	Reference
Luminal A	ER+ve and/or PR+ve, Her2–ve, any CK5/6/Her1	[15-17]
Luminal B	ER+ve and/or PR+ve, Her2+ve, any CK5/6/Her1	[15-17]
Basal-like (triple –ive)	ER–ve, PR–ve, Her2–ve, CK5,6+ve and/or Her1+	[18]
Her-2+over-expressing (ERBB2)	ER–ve, PR–ve, Her2+ve, any CK5/6/Her1	[19]
Normal Breast –like	"unclassified" (negative for all 5 markers), displays putative-initiating stem cell phenotype	[20, 21]
"claudin-low" group	"unclassified" (negative for all 5 markers), displays putative-initiating stem cell phenotype, Often triple negative, displays low expression of cell-cell junction proteins and e-cadherin, frequently infiltration with lymphocytes	[20, 22]

Positive (+ve), negative (–ve), estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptors (EGFR) 1 and 2 (Her 1 and 2) and cytokeratin 5, 6 (CK5,6).

Table 1. Distinct Molecular Tumor Categories of Breast Cancer and their Receptor Status

However, most investigators use the presence (+) or absence (-) of immunohistochemical (IHS) markers or receptor combinations that are expressed by neoplastic cells. In this manner, distinct tumor categories to-date are identified by the expression of estrogen receptors (ER), progesterone receptors (PR), human epidermal growth factor receptors (EGFR) 1 and 2 (Her 1 and 2) and cytokeratin 5, 6 (CK5,6) [15-19, 23-25]. Generally, molecular sub-types correspond to IHC receptor status [26]. These subtypes are of great clinical and research importance as they are utilized to administer and target therapeutic regimes based on predictions of response. Furthermore, these subtypes have been shown to display a wide variety of responses to different treatments [27-30] and are associated with other clinical outcomes, such as patient relapse and overall survival. The most favorable outcomes are noted for the luminal A subtype, which are hormone sensitive [31]. The Her2+ and basal subtypes are noted as more aggressive and have fewer therapeutic options [31-33]. The normal-like and claudin-low are unclassified (negative for all major receptors), and associated with poor prognosis [20-22, 30, 34]. Several small studies support the concept that molecular subtype and tumor receptor status may change during ordinary disease progression and a major study completed by MacFarlane *et al*. in 2008 revealed that 21% of relapsed tumors had changes in either ER/PR or HER2 receptor status [35]. This significant proportion led the author to suggest that biopsies of relapsed/ metastatic breast cancers should be performed routinely. This also should be an important consideration in research. In summary, treatment options considered effective in primary stage cancer may no longer be optimal in later stages of the disease, including relapses and metastasis as determined by the current classification scheme.

2.3. Other classification approaches

Other breast cancer classification approaches are also used to assist in both prognostic and treatment decisions. These include computer models that are based on a combination of several factors and offer individual survival predictions and calculations of treatment benefits [36]. For example, patients undergoing systemic adjuvant therapy can determine optimal treatment through the commercially available computer model *Adjuvant* which has been successfully validated in several cohorts, including the United States and Canada [36, 37]. Other useful classification tools utilized for breast cancer treatment choices include prognostic assessments (such as USC/Van Nuys prognostic index (VNPI)) and general comorbidity assessments [38, 39]. Also consider the case of familial breast cancer (genetic classification) whereby a patient may opt to undergo preventative measures such as mastectomy. Additionally, immunohistochemistry testing, other than those mentioned earlier, continue to prove favorable as prognostic markers across various molecular subtypes [40]. For example, in human breast cancer epithelial cell proliferation is considered a significant prognostic marker [41] and could possibly be used as a prediction tool to measure different hormone treatment related risks [42]. Therefore, the immunohistochemical marker, Ki67 (a nuclear protein expressed by cells in all active phases of the cycle except for quiescent or resting cells) is utilized frequently to evaluate proliferation [43]. Such labeling indicates a significant association with higher carcinoma grade, clinical response to endocrine therapy, higher risk of relapse, and worse survival in patients with early breast cancer [44].

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3. Cell of origin

While scientists have predicted that specific breast cancer types may arise from different types of progenitor cells, it has been difficult to identify the cells of origin, and the topic remains controversial to date. Breast tumors represent a heterogeneous collection of cell populations with different biological properties [45]. Shared molecular features have made it difficult to distinguish the cell populations of breast cancer tumors and only recently have researchers been able to differentiate stem cells from other progenitor cells. Within this context, tumor evolution has been explained by two main theories. The traditional clonal evolution model is based on the premise that all tumor cells have the capacity to undergo self-renewal which is an indication of their potential to undergo tumor progression and drug resistance [46, 47]. The Cancer Stem Cell (CSC) theory emphasizes the ability of only a minor population of tumorigenic cells capable of self-renewal and differentiation [20, 48] and this specific sub-set of CSC's gives rise to new tumors which are phenotypically identical to the original tumors [49]. CSC's are believed to be a small population of cells with dysregulated self–renewal properties capable of continuous self-renewal and differentiation and responsible for tumor existence treatment resistance and relapse. Existence of CSC's in various types of tumours, including breast cancer [50],[51] has been identified. ALDH (aldehyde dehydrogenase)-1 is a marker of normal and malignant human mammary stem cells [52], and these cells can also be isolated using the cell surface markers epithelial-specific antigen (ESA), CD44 and the absence of the expression of CD24 [53]. When transformed cells undergo epithelial-mesenchymal transition (EMT), they have been noted to gain properties of stem cells [54]. Although evidence supporting the CSC model was initially obtained from acute myeloid leukemia [55], successive studies maintain that solid tumors, including breast cancer tumors, are also driven and sustained by CSCs [50].

Regardless of origin, an abundance of research has clearly confirmed the existence of cancer stem cells (CSCs) or tumor-initiating cells (TICs) in a variety of human cancers [55-58], including breast cancer [50]. Nonetheless, most of the therapeutic approaches available, inclusive of chemotherapy and radiation, lack the ability to effectively kill these populations [59-62]. This may explain the lack of progress in eliminating cancerous tumors and preventing metastasis and may help to rationalize therapeutic resistance; therefore, the CSC or TIC population has become a target for cancer prevention and therapy [63]. A general consensus exists in the literature that breast cancer re-occurrence is assumed to be caused by a subpopulation of tumor initiating cells possessing stem cell attributes of a tumor as well as resistance to chemotherapy, radiation and other forms of treatment [64-66]. Of great interest is the role of CSC's in tumor relapse and resistance to therapy and recent articles suggesting that such resistance can be overcome.

4. Breast cancer progression models

The combined research efforts of various scientific disciplines have resulted in the development of a disease progression model for breast cancer (See Figure 1) and include a continuum of lesions through to invasive carcinoma and eventually metastatic disease [30, 48, 67, 68]. For decades, it was thought that metastatic dissemination occurred as a final step in cancer and

was the responsibility of genetic changes of malignant cells in the primary tumor [69]. In 2008, Husemann *et al*, used transgenic mice to show systemic dissemination (specifically to lungs and bone marrow) of mammary tissue derived premalignant cells prior to the emergence of mammary tumors [70]. Additionally, this research reported that systemic dissemination of tumor cells can occur in pre-invasive stages of tumor progression as observed in female patients with ductal carcinoma *in situ*. A complementary accumulation of evidence supports the evolution of an early dissemination model, where malignant cells outside the primary lesion can also migrate to distal sites (such as lung and bone marrow) and cause tumors via various genetic programs [54, 71, 72]. Such a model is inclusive to "self-seeding", the term coined when cancer cells not only seeds regional (lymph nodes) and distant sites but also fuel the growth of the original tumor itself [73].

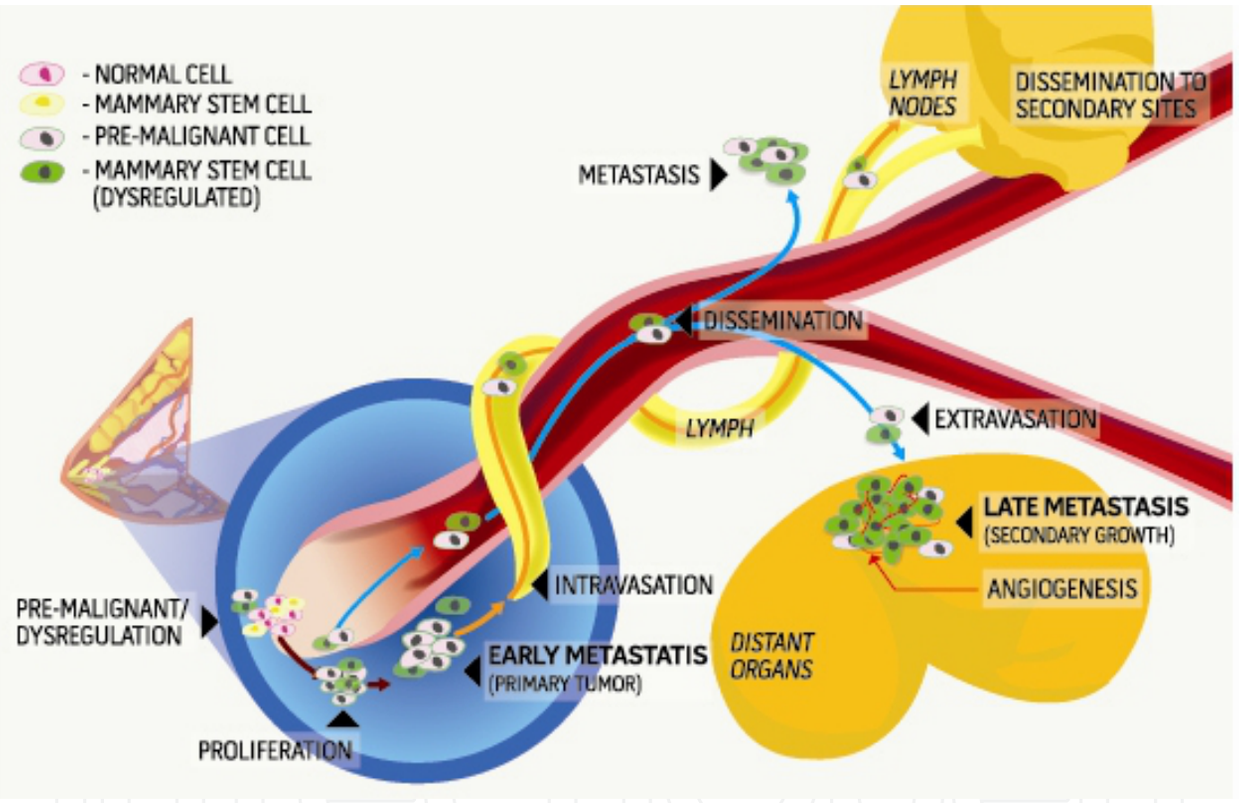

Figure 1. (Tobin, GA, 2011) Physiological Aspects of Disease Progression in Breast Cancer: The development of breast cancer has been proposed as a multi-step process. The most deadly aspect of breast cancer is metastasis and involves a cascade of reactions. Although the molecular mechanisms underlying this process are not fully understood, any disruption along the cascade could arrest disease progression.

Thus, breast cancer is not a single disease, but rather an assortment of diseases with diverse characteristics and clinical outcomes which will likely always require a variety and/or combination of treatments or alternatively, a broad spectrum application. Combine this with the fact that, despite major advances in our understanding of the biology of cancer, further research is required to improve our understanding of tumor establishment, progression and dissemination - the principal cause of mortality. Then, our goal in breast cancer research

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regarding treatment would be to identify novel therapies and maximize, based on our current understanding of the disease, the use of such in preventing and treating as many aspects of the disease as possible.

5. Molecular and endocrine controls

5.1. BRCA and other gene expression signatures associated with increased risk of breast cancer

Less time, low cost and new sequencing technologies are all accomplishments that yield impact on molecular biomedical research. Recently, for example, such advances (combined with some prospective epidemiology studies) have allowed researchers to compare the DNA from healthy breast tissue, initial tumor cells and then cells obtained nine years later when the breast cancer had metastasized [74]. The 32 DNA mutations reported in the metastasized cells are a prime example of how technological advances can serve to provide us with ample data regarding gene expression that may offer insights into the progression of breast cancer disease. However, consider that gene expression profiling / signatures of primary breast tumors can only be used as a predictor of susceptibility or disease progression in breast cancer, particularly metastasis. Currently, it is not possible to accurately predict the risk of metastasis or prevent it. As a result, more than ½ of the patients treated with adjuvant chemotherapy are needlessly exposed to harmful side effects [5]. This presents another compelling reason to further study drug targets that are specific to, and have potential for, treatment in metastatic breast cancer.

Nonetheless, based on current knowledge, and aside from the many identified factors that could impact the risk of developing breast cancer (including personal and environmental), genetic mutations in critical cancer genes (both tumor suppressor and oncogenes) have been identified for their increased or associated risk with breast cancer [75]. Of the many that have been reported throughout the history of cancer genetics, Table 2 below captures those that stand out principally for their research and/or clinical significance in relation to breast cancer and summarizes major function, encoded proteins and known disease associations or risk factors. Such genetic aberrations are acquired over a person's lifetime; or less commonly, are inherited. While it is estimated that only 5% to 10% of breast cancers are hereditary, some gene variations are associated with both hereditary and somatic mutations [75]. The tumor suppressor genes *BRCA1* and *BRCA2* are the major genes related to hereditary breast cancer [76], and mutations in these and other *BRCA* genes in women are associated with a 60-80% risk of developing breast cancer throughout their life span [77]. Other genes with inherited alterations, including *CDH1*, *PTEN*, *TP53*, *CHEK 2*, and *ATM* have been noted to increase or are associated with the risk of developing breast cancer [78]. Most notably, the latter three have presented the strongest evidence related to the risk of developing breast cancer [79]. Somatic mutations mostly reference *ERBB2/HER2(neu)* in breast cancer, however *TP53* genes and others have been associated with some cases of breast cancer in this manner [80]. It is noteworthy that not all people who inherit mutations in these genes will develop cancer.

Gene	Major Function: Associated Proteins	Risk Factor	Ref.
BRCA1 (BReast CAncer gene one)	Encodes breast cancer type 1 susceptibility protein; responsible for DNA repair, transcriptional regulation and cell cycle check point control.	Strong evidence indicating a 60 – 80% risk of developing breast cancer for people with mutations.	[81 - 86]
BRCA2 (BReast CAncer gene two)	Encodes BRCA2 susceptibility protein involved in the repair of chromosomal damage, especially in the error-free repair of DNA double strand breaks. As with BRCA1, indicates a high degree of risk.	Reduced levels of the BRCA2 protein may cause Fanconi anemia. Patients with such are prone to several types of cancers, including reproductive system associated tumors.	[87] [88]
ATM (Ataxia telangiectasia mutation)	Encodes protein with a phosphatidylinositol 3-kinase (PI3K)–like domain which plays a central role in the complex processes that repair DNA double-strand breaks. Also involved in regulation of cell cycle progression and the maintenance of genomic stability.	Confers susceptibility and linked to an modest increase (up to 2 times) of breast cancer.	[89-93]
p53 (TP53) (Tumor Protein p53)	Encodes p53 protein and regulates cell cycle, preventing tumor growth.	Causes Li-Fraumeni syndrome: results in higher-than-average-risk of breast cancer and several other cancers.	[94-96]
CHEK2 (Checkpoint kinase 2)	Encodes a serine/threonine-protein kinase which plays a critical role in DNA damage signaling pathways. Phosphorylates and regulates the functions of p53 and BRCA1.	Causes Li-Fraumeni syndrome. Can double breast cancer risk.	[97-99]
PTEN (Phosphatase and tensin homolog)	Encodes phosphatase and tensin homolog protein, namely phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase, which is involved in the regulation of the cell cycle.	Causes Cowden syndrome which presents a higher risk of both benign and cancerous tumors in the breast, digestive tract, thyroid, uterus, and ovaries.	[100, 101]
CDH1 (Cadherin 1 Gene) or E-cadherin (epithelial cadherin)	Encodes E-cadherin protein. Down-regulation decreases the strength of cellular adhesion, increases cellular motility; allowing cell invasion.	Increased risk of breast cancer, particularly invasive lobular breast cancer.	[102-104]
** ERBB2, (HER2/neu), or VEGF	Encodes a transmembrane receptor with constitutive tyrosine-kinase activity.	Amplification occurs in 15-30% of human breast cancers.	[34] [80]

** ERBB2 (erythroblastosis oncogene B), HER2/neu (Human Epidermal growth factor Receptor2, neu: derived from a rodent (neu)ral tumor), VEGF (vascular endothelial growth factor).

Table 2. Familiar Genetic Mutations, Encoding Proteins and their Major Functions in Relation to the Associated and Increased Risk of Developing Breast Cancer.

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Those listed in Table 2, as mentioned, represent familiar genes associated with breast cancer. However, others genes that are applicable in breast cancer progression will be reviewed in terms of their associated cell signaling pathways. Considering the profound implications that the CSC theory has for cancer chemoprevention and therapy, combined with our interest in plant based molecules, we will also examine gene function in the context of opportunities for natural product compounds in CSC self-renewal.

5.2. Endocrine controls

Over the years, and more notably since the discovery of suitable breast cancer cell lines and animal models, the symbiotic relationship between lab research and clinical investigations have advanced our knowledge of endocrine action. Breast cancer is influenced and highly regulated by several sex and growth hormones (including estrogens, androgens, progesterone, prolactin and insulin-like growth factors) and each of the sub-types and gene expression patterns of breast cancer are characterized by both unique and specific endocrine controls [105]. Particularly, estrogens and progesterone have received the greatest attention likely because of their involvement in normal and neoplastic mammary tissue and the scale of their associated risk estimation for breast cancer. Research into the role of androgens has likely been a consideration in breast cancer research as androgens are necessary precursors to all endogenous estrogens. The role of prolactin in the pathogenesis of breast cancer remains unclear given the scarcity of studies to date. Similarly, although well studied, the role of Insulin-like growth factor (IGF) in breast cancer is inconsistent.

There are challenges in defining the role of progesterone in breast cancer and the role of progesterone receptor (PR) action in breast cancer remains divisive. In breast cancer, progesterone has biphasic effects (both proliferative and inhibitory) on breast cancer cell lines grown *in vitro* [106, 107]. Depending upon cellular context and/or the presence of secondary agents, there may be a role for progesterone as a priming agent with growth promoting activity [106].

While progesterone is found as a single hormone, the major endogenous estrogens in females include estrone (E1), estradiol (E2), and estriol (E3) and are primarily produced during menopause, in non-pregnant and pregnant females, respectively [108]. A variety of synthetic (xenoestrogens) and natural substances have been identified that also possess estrogenic activity including those derived from plant products (phytoestrogens) and fungi (mycoestrogens) [108]. The actions of estrogens are mediated by their respective receptors binding to specific DNA sequences to activate the transcription of estrogen receptor (ER)-regulated genes, including direct target genes [109]. Approximately 80% of breast cancers demonstrate expression of PR and/or ER [110]. Once established, these breast cancers which are classified as either hormone-sensitive or hormone-receptor-positive cancers are reliant on hormones to grow. Thus, treatment includes the suppression of hormone production in the body for these specific breast cancers. Therefore, this section will provide an overview of estrogens, their past indications in the treatment of breast cancer and their potential role in relation to naturally occurring dietary compounds.

5.2.1. The role of estrogens and breast cancer

The link involving estrogen and breast cancer can be traced back to as early as 1896 when a surgeon in England reported an improvement in condition in three young female breast cancer patients after the removal of their ovaries [111]. Since estrogen had not yet been discovered, the surgeon had unknowingly removed the source of estrogen that promotes the survival and division of cancer cells [111]. Since that time, a lot of evidence has shown that interactions between estrogens and their receptors influence the pathogenesis of breast cancer. Estrogen promotes proliferative effects on cultured human breast cancer cells [112]. Estradiol affects breast cancer risk by controlling the mitotic rate of breast epithelial cells and high levels of estradiol in post-menopausal women are also known to increase the risk of breast cancer [113]. Estradiol has also been shown to increase breast cancer risk via its metabolite, catechol estrogen 4-hydroxyestradiol, causing direct DNA damage through the formation of free radicals [114]. Estradiol has also been shown to modulate breast cancer cell apoptosis [115]. ER is a major determinant of the cellular response of estrogen and has been indicated in breast cancer promotion [116, 117]. The binding of estrogen to the ER modulates the transcription of a series of genes, including those coding for proliferation.

The close relationship between the etiology of breast cancer and exposure to estrogen warrants examination of key variables that may affect estrogen homeostasis, in particular those exhibiting anti-estrogen activity. Because of their impact on the primary and metastatic aspects of disease, anti-hormonal drugs are the mainstay breast cancer treatment. The goal in treating hormone receptor +ve breast cancers is to utilize drugs which suppress production of estrogen in the body. Estrogens in naturally occurring dietary compounds such as soy are used as an alternative to hormone therapy because of their anti-proliferative effects. This practice, in breast cancer treatment, is widely known as hormonal therapy, or anti-estrogen therapy, but is not representative of the term hormone replacement therapy [118].

6. *In vitro* and *in vivo* breast cancer models and their relevance to human disease

In 2007, Vargo-Gogola and Rosen summarized appropriately, within their title, the challenge of modelling breast cancer; "One size does not fit all"[31]. Considering the heterogeneity nature of this disease, these researchers rather addressed the reasonable question of the most powerful way to investigate breast cancer with respect to cell lines and animal models. We agree with their conclusion that an integrated and multi-systems approach is the strongest way to model this disease. Here, we briefly discuss the most commonly utilized *in vivo* and *in vitro* models and their relevance in human breast cancer.

6.1. Mammary carcinoma cell lines

Our current knowledge of breast cancer is mainly based on *in vivo* and *in vitro* studies completed with breast cancer cell (BCC) lines. The first BCC line, namely BT-20, was established in 1958 [119] and since then the number of permanent lines sustained are relatively low despite continuous research in breast cancer [120]. In fact, the number of commercially available BCC

lines (ranging from 70 – 80 based on ATCC and similar commercial suppliers) represent mostly cell lines that were established more than 25 years ago. It has been suggested that this inefficiency in producing new and improved lines is likely due to technical difficulties in extracting viable tumor cells from their neighboring stroma [120]. In the past, it has been estimated that more than two-thirds of all scientific abstracts related to studies on mentioned BCC lines include the MCF-7, T-47D, and MDA-MB-231 lines [120]. Although no single cell line is entirely representative of human breast cancer, cell lines have been widely used. As a result, we have a greater understanding of breast cancer biology. Additionally, these cell lines have advanced the ability of pre-clinical models to predict pharmaceutical activity and therapeutic applications for further study. Despite this, the use of cell lines for application in human disease is limited. The single most significant question, considering the devastating effects or outcomes of metastasis, is the issue of whether these cell lines are representative of metastatic conditions, or contain tumor initiating subpopulations. There are studies to indicate distinctions between tumor and non-tumor initiating populations at the cellular and molecular level [31, 121]. Research indicates that the two luminal subsets (A and B) evident in tumors are not apparent in the cell lines, and the basal-like cell lines are actually representative of two distinctive clusters (A and B) that are not apparent in analyses of primary tumors [16]. The differences in cell culture may be due to the absence of stromal or physiological interactions and/or signaling [122] [123]. However, there is evidence that cell lines may be derived from subpopulations of tumor cells that are selected because they grow well. For example, it has been noted that differences between the genome aberration patterns for the basal-like and luminal clusters in the cell line system don't match differences in these subtypes in primary tumors. Furthermore, the highly invasive Basal B cells carry the distinctive phenotype associated with the subpopulation of tumorigenic stem cells identified in breast cancer [50, 124]. Also, one must consider the issue of variants of the same cell lines and the phenotypic changes that can occur due to environmental exposures, rate of passage and age of cells.

Perhaps the most highly debated topic regarding the use of breast cancer cell lines revolves around the MDA-MB-435 cell line, and by extension MDA-MB-435S (ATCC® HTB-129™) and derivatives. Spontaneously metastatic and originally designated as Basal B and being derived from a breast carcinoma, the MDA-MB-435 cell line has subsequently been questioned because of recent evidence indicating that it might originate from melanoma or may have been cross-contaminated with the M14 melanoma cell line [125-130]. While some researchers continue to use this cell line as a breast cancer mouse model [131] and conclude that this cell line is in fact breast cancer cells [132], others insist that the MDA-MB-425 cell line is a melanoma cell line and renders its use as improper in breast cancer studies [133]. Later in the chapter, we will describe some research that has been conducted using MDA-MB435 cell lines and/or its derivatives as breast cancer models in research involving natural, despite this controversy. This may not be all bad news for natural product researchers. On the bright side, natural product therapies indicated in the controversial cells line MDA-MB-435 and its derivatives then point toward activity in melanoma as opposed to breast cancer.

Nonetheless, there is clearly a need for new cell lines that are representative of all the known sub-types of breast cancer. Currently, researchers have the option of using a group (or panel)

of subtype cell lines to increase power in their research, as proposed by Neve [2006] [129]. To date, and based on our knowledge, there has been no evidence of a male breast cancer derived cell line. With the incidence of male breast cancer rising [2], relevant models require further development.

Cell lines are easy to culture, inexpensive in comparison to animal models, and provide an unlimited source of homogenous material to work with. Comparisons between researcher and studies are usually fairly consistent and reproducible. A limitation of cell cultures is their inability to measure tumor-stromal cell interactions, however the growth of cell lines for transplantation allows for such *in vivo*.

6.2. Animal models

Animal models have allowed researchers to gain much insight into the disease progression, in particular metastasis. In addition to advancing the concept of metastasis [134, 135], our knowledge of the biological function of genes and signaling pathways has progressed as a result of the vast amount of information generated from animal models. Experimental systems based on mouse models that are currently used to study breast cancer can be categorized as tumor transplantation and genetically engineered mice (GEM) – often termed as transgenic. Here we will focus on commonly utilized models within each of these categories and discuss some of the advantages and limitations of each in relation to human breast cancer disease relevance.

6.2.1. Transgenic or (GEM) models

Different GEM breast cancer models are useful for studying distinct signaling interactions and for testing therapies that target pathways involved. Genomic deletion of tumor suppressor genes or the transgenic insertion of oncogenes allow these mice to be a relevant tool to investigate the spontaneous initiation of breast tumors in each step of metastasis [136]. Specific to breast cancer, the expression of oncogenes in explicit breast regions are restricted via a mammary gland specific promoter [136]. MMTV (mouse mammary tumor virus) or WAP (Whey Acidic Protein) promoter control the expression of oncogenes (i.e: PyMT, ErbB2, Wnt1, or Ras) in transgenic mice and initiate mammary gland tumors which lead to metastasis in various organs [137]. The MMTV-PyMT transgenic mouse model represents hyperplasia, adenoma, and early or late carcinoma as seen in human cancer stages. In breast cancer, this model demonstrates short latency tumor development specific to the mammary gland with a high incidence pulmonary metastasis [138]. The deletion of mammary epithelial cell-specific tumor suppressor genes results in similar conditions observed in human cancer patients, including spontaneous tumors, bone metastasis, loss of estrogen receptor (ER) expression, and hormone-responsiveness [139, 140]. Crossing GEM mice with other transgenic mice has allowed researchers to investigate the role of genes and signaling pathways in tumorigenesis and metastasis. While tumor progression was delayed in MMTV-PyMT or MMTV-ErbB2/neu mice crossed into an Akt or PTP1B-deleted genetic background [141, 142], increased metastasis was observed in the MMTV-PyMT mouse in a CD44_/_ background; the later indicates the importance of receptors and other secreted factors in epithelial–stromal interactions or overall

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tumor environment [139]. In this regard, much insight has been gained through the use of GEM models to indicate the role of both innate and adaptive immune responses in the progression of breast cancer. For example, pulmonary metastasis is notably diminished via the selective loss of the interleukin (IL-) 4 cytokine in PyMT/IL-4_/_ mice through inhibition of IL-4 mediated EGFR signaling of mammary tumors [143]. The understanding of such interactions are important if we consider that tumor-associated immune cells release numerous growth factors, including cytokines, chemokines, and enzymes that promote tumor growth, angiogenesis, and metastasis, and thus are associated with poor prognosis [136]. Inducible GEM mouse models (those capable of expression or repression of certain genes) have advanced knowledge with respect to the association of multiple genetic mutations of oncogenes in relation to tumor establishment and maintenance [144]. There are several drawbacks to consider in the use of GEM models. There are inherent differences in mouse and human, for example: BRCA1 and p53 are on the same chromosome in mice, but not in humans [136, 144]. Technical discrepancies, depending on methods used to generate the model and the interpretation of results can present varying outcomes. Also, temporal and spatial tumor development and metastasis differ depending on the mouse strain used. For example the percentage of ER/progesterone receptor (PR)+ve tumors in Wap-Cre/Trp53_/_ mice are high while MMTV-Cre/Trp53_/_ mice report a low incidence of such [145]. The molecular profile of mammary tumors from GEM models represent the subtypes of human breast cancers, including those included in Table 1, however no single model to date is representative of all expression patterns and characteristics of human cancer.

6.2.2. Tumor transplantation model

Syngeneic transplantation refers to the transfer of cancer cells from one mouse into another with identical genetic background [146] and such models are used to establish organ specific metastasis [147]. The 4T1 breast cancer tumor model (a syngeneic mouse model) is considered excellent for testing experimental cell-based immunotherapy strategies and is comparable with stage IV human breast cancer [148]. The injection of 4T1 cells, originally derived from a spontaneous mouse mammary tumor are injected into the mammary fat pad of a syngeneic animal and the rapid proliferation of cells forms tumors and eventually metastasizes to the lungs, liver, bone and brain [146, 147, 149]. Furthermore, this model has been refined through the development of 4T1 cell lines to employ varying degrees of metastasis that are location specific and representative of distinct gene expression signatures [150]. Xenograft transplantation encompasses the use of human cancer cells into immuno-compromised mice via intravenous, intraperitoneal or subcutaneous injection, orthptopically or ectopically [136, 151]. First discovered in 1962, nude mice have been commonly used for xenotransplantation because they lack a thymus and are unable to mount most types of immune responses, including rejection of allografts and xenografts [152]. Severe Combined Immunodeficiency (SCID) mice present with impaired ability to make T or B lymphocytes and are used as model organisms for research into transplantation strategies as they cannot reject tumors and transplants [153]. In general, rodents with immunological defects are typically resistant to growth of mammary carcinomas, and despite improvements these approaches have yielded low percentage of successful breast tumor engraftment compared to other types of cancers

[154]. Recent mouse models have contributed significantly to the understanding of breast cancer, however further research into suitable animal models will be required to advance development of new therapies for breast cancer. The growth and metastasis of human breast cancer cell lines *in vivo* allows the measurement of gene function relative to disease progression and has provided much insight into the use of investigational drugs intended interrupt or interfere with tumor growth [155].

Table 3 provides an overview of the defining characteristics of established breast cancer cell lines (mouse and human) commonly used in both *in vitro* and transplantation models.

Cell line	Species	ER Status	PR Status	Metastasis Location	Ref.
4T1	Mouse	+	+	Lymph node, blood, liver, lung, brain, bone	[149]
BT-474	Human	+	+	Bone	[156]
FII3	Mouse	+	+	Lung	[157, 158]
MCF-7	Human	+	+	Lymph node, lymphatic vessel	[159, 160, 161]
MDA-MB-231	Human	–	–	Lung, liver, brain and bone	[162, 163]
MDA-MB-435	Human	–	–	Lung	[164]
MDA-MB-453	Human	–	–	Bone	[165]
SUM1315	Human	–	–	Lung, bone	[166, 167]
SUM149	Human	–	–	Lung	[167]
T47D	Human	+	+	Lymph node, lymphatic vessel	[168]

Table 3. Breast Cancer Cell Lines for *in vitro* and transplantation models

It is unlikely that any one transplantation model will ever replicate the complexity of the whole cancer process; however studies to date demonstrate that xenographs are relevant in human breast cancer. Treatment with Herceptin was shown to improve the anti-tumor activity of paclitaxel and doxorubicin against HER2/neu-overexpressing human breast cancer xenografts leading to consecutive favorable clinical trials [169, 170]. Davis *et al.* [2004] reported the effective inhibition of tumor growth and metastasis in an orthotopic xenograft model by the use of combination therapy of paclitaxel and neutralizing antibodies targeting vascular endothelial growth factor receptor 2 (VEGFR2) [171]. The results of this research likely led to the development of bevacizumab, a humanized monoclonal antibody that targets vascular endothelial growth factor A (VEGF-A) [172]. Although, bevacizumab was removed as a breast cancer indication by the FDA [173], this is yet another example of how transplantation models lead to further development in the clinic. While these are merely a few cases for breast cancer and the use of xenograft studies, the information obtained from such has been translated into successful clinical trials for a variety of cancers [174-178]. Furthermore, useful information has been gained from transplantation models with respect to toxicity and in the identification of predictive biomarkers.

In spite of these successes, the xenograft model presents disadvantages in its ability to predict clinical response to therapy. Most animal tumors do not accurately model clinical metastatic disease. The use of immuno-compromised mice prevents an immune rejection response which is fundamentally different from the human system where the immune response promotes primary growth of tumor cells and their migration to secondary organs [136, 179]. Most xenograph models do not metastasize at the common sites of human breast cancer (such as lymph nodes, liver, bone and brain) as they prefer to colonize in the lungs [5]. However, improved models that have been created to represent human breast cancer metastasis from a primary orthotopic site to human bone, such as initially published in 2005 by Kuperwasser *et al* [167]. Consider that some xenograph models which utilize subcutaneous injection of tumor cells into the flank and mammary fat pad are not as representative of clinical disease as those that use orthotopic transplantation of cells into the mammary gland. Inherent discrepancies in the background of mice and humans should be considered, and this is of particular importance in predicting side effects for cancer therapy targets [180]. Additionally, the area of metastasis can change depending on the cells line utilized or methods of inoculation [167]; thus, there are considerable technical issues. One could overcome such restrictions by use of clinical isolates. However the utility of primary xerographs is inefficient; access is limited, results are restricted by sample size and thus, studies to date indicate only some degree of success [181]. Current research with aims to generate partial human immune systems or intact populations of human cells in mice systems may also overcome such limitations. We must consider that despite all advances to date, animals do not represent a complete model of human disease. For example, tumor relapse would be especially problematic to study as the usual life span in the majority of mice does not exceed two years. Aside from these underlying basic issues, there have been issues associated with the testing of natural products within these models. Further clarity on the fundamental mechanisms of tumor progression and metastasis and new drug targets will be unveiled as mouse models advance. Until then, we will continue to rely on current disease models and choose such based on disease state and therapeutic targets.

7. Breast cancer signaling pathways

Each of the identified breast cancer subtypes and gene expression patterns are dependent on different oncogenic pathways [105, 182]. The maintenance and differentiation of normal breast tissue is controlled by many signaling pathways and involves cytokines and chemokines, growth factors, steroid hormones, integrins, adhesion molecules and their respective receptors [183]. The regulation of such by single or combined components of the tumor microenvironment (such as fibroblasts, macrophages / lymphocytes, endothelial cells, vessels and proteins of the extracellular matrix (ECM) and stroma) have been implicated in various ways in the promotion, growth invasion and metastasis of breast cancer [184]. Cross talk or communication between the cancer cells and the factors within the tumor environment, including secretion factors from the tumor itself, can modify expression and signaling [184]. Of the several pathways indicated to play a role in cancer and CSC self-renewal, Notch, Wnt/Beta(β)-catenin,

and Hedgehog (Hh) have been identified in human mammary cancer [124, 185, 186]. Additionally, evidence has mounted to strengthen the link between nuclear factor kappa-B (NF-κB), stem cells and breast cancer as elegantly reviewed in a recent paper by Shostak and Chariot (2011) [187].

7.1. Notch pathway

Four Notch proteins, namely Notch-1 to Notch-4, are expressed as transmembrane receptors in a variety of stem/progenitor cells [188, 189]. The binding of specific surface-bound ligands are responsible for triggering cleavage events at the Notch proteins by ADAM (A Disintegrin and metalloproteinase domain-containing protein) protease family and γ-secretase [188-191] causing the intracellular domain of Notch to be released and translocate to the nucleus. Once in the nucleus, downstream target genes (including c-Myc, cyclin D1,p21, NF-κB) are activated [190, 192-197]. Known for their ability to modulate the development of various organs and control cell proliferation [198], the notch activated genes and pathways have been reported to drive tumor control through the expansion of CSCs [198-202]. This associated role in self-renewal function of malignant breast cancers CSCs [198], combined with the fact that Notch inhibitors can kill breast cancer cells *in vitro* and *in vivo*, may partially explain why Notch expression and activation has been associated with a poor prognosis in mammary carcinomas [203-205]. In fact, research findings in breast cancer have presented compelling reasons to target Notch as a therapeutic target in solid tumors. In addition to its ability to regulate survival and proliferation in bulk cancer cells [205] and CSCs [206-209], notch plays a pro-angiogenic role in tumor endothelial cells [210, 211]. Farnie *et al* reported that activated Notch-1, Notch-4, and Notch target Her-1 expression in ductal carcinoma mammospheres *in situ* samples, but not from normal breast tissue [206, 212, 213]. Inhibition of Notch with a gamma-secretase inhibitor (GSI) or a neutralizing Notch-4 antibody has been reported to reduce the ability of ductal carcinoma in situ-derived cells to form mammospheres [207, 214]. Such results suggest that Notch inhibition may have significant therapeutic effects in primary lesions, may be able to preferentially target breast CSCs (responsible for reoccurrence and metastatic disease) and counteract angiogenesis [213]. Cross talk with the NF-κB pathway and Notch1 have been reported in a variety of cell interactions [192, 215-219], including the stimulation of NF-κB promoters[217] and the expression of several NF-κB subunits [192, 215-220].

7.2. Wnt/β-catenin pathway

The canonical (Wnt/β-catenin) pathway, including Wnt-1, -3A and -8 is likely the best characterized and traditionally defines Wnt signaling, however other pathways have been described including a non-canonical (planar cell polarity) pathway (including Wnt-5A, - 11) and the Wnt/Ca2+ pathway (protein kinase A pathway) [221-224]. Although it has been more than 25 years since the discovery of the Wnt gene, its structure remains unknown and signaling pathways are not well defined, especially those independent of β-catenin. Perhaps this challenge can be somewhat explained by the recent discovery that differences in cell signaling outcomes may be attributable to precise pairings of Wnt ligands with analogous cellular receptors [225]. For example, if we consider that the mammalian genome codes for 19 Wnt proteins and 10 Fzd

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receptors, there are potentially 190 Wnt/Fzd pairing combinations [226]. Although all of these ligand/receptor pairings have not been unveiled, we already know that the Wnt/β-catenin pathway has been established for its ability to alter cell proliferation, migration, apoptosis, differentiation and stem cell self-renewal [224, 227-230]. The essential mediator of the canonical pathway is β-catenin, and its two known distinct functions are based on cell specific locations. Accumulation of β-catenin within the cytoplasm leads to activation of Wnt target genes such as c-Jun, c-Myc, fibronectin and cyclin D1 [186, 231-236]. Prior to nuclear translocation, β-catenin operates in the membrane to maintain cell–cell adhesion via cooperation with the epithelial cell–cell adhesion protein E-cadherin [223]. The Wnt signaling pathway is activated via the binding of ligands to transmembrane receptors encoded by the Frizzled (Fzd) gene family and in conjunction with co-receptors, such as low-density lipoprotein receptors (protein 5 and 6) [237] This Wnt-Fzd interaction results in dephosphorylation, accompanied by decreased levels of degradation and causes the accumulation of β-catenin in the nucleus [231]. In the absence of Wnt signaling, β-catenin is quickly degraded in the cytoplasm. Without Wnt signaling, phosphorylation of adenomatous polyposis coli (APC) [238] via a cytoplasmic destruction complex results in ubiquitination of β-catenin which is then prone to proteasomal degradation [231]. Additionally, nuclear levels of β-catenin are lessened by their interaction with APC and Axin, both known for their function in transporting β-catenin back to the cytoplasm. In the nucleus, transcriptional corepressors interact with DNA-binding T-cell factor/lymphoid-enhancer factor (Tcf/Lef) proteins, such as Groucho/TLE, and are enabled to block target-gene expression when β-catenin is held at low levels. [239-242]. Wnt binding to the Fzd or low-density lipoprotein receptor protein-membrane receptors results in the accumulation and stabilization of translocated (from cytoplasm to nucleus) β-catenin [237]. Inhibition of such interactions has been noted by secreted Fzd-related proteins, Dickkopfs, and Wnt inhibitory factor-1 (WIF-1) [243, 244].

Since the initial observation that Wnt overexpression results in malignant transformation of mouse mammary tissue [245], aberrant regulation of the Wnt signaling pathway has emerged as a prevalent theme and continues to develop as a fundamental mechanism in broad cancer biology [246]. While Wnt pathway mutations (genetic and epigenetic) are rare in mammary carcinoma, overactive Wnt signaling has been noted in the majority of breast cancers, including rare classes (i.e.: triple -ve type) via several potential mechanisms [145, 233, 247-256]. Several studies to date have indicated that the expression of both Wnt receptors and their ligands are characteristic of breast cancer and furthermore certain receptors and ligands may be breast cancer type specific. In 2004, Bafico *et al*. reported autocrine Wnt signaling in a panel of breast cancer cell lines, including MDA-MB-231, which were identified by the presence of unstabilized β-catenin and then subsequently reduced upon expression or by the addition of the soluble Wnt inhibitors sFRP1 or DKK1 [257]. The expression of the Wnt receptor FZD7 is characteristic of certain rare types of breast cancer [258]. Additionally, the knockdown of FZD7 in cell lines representative of triple -ve breast cancer reduced the expression of Wnt target genes, inhibited tumorigenesis *in vitro* and greatly retarded the capacity of the MDA-MD-231 cell line to form tumors in mice [246, 259]. With respect to Wnt ligands, secreted frizzled related protein (sFRP)-1, an effective competitor and binding site with FZD receptors for Wnt ligands, has been shown to be ectopically expressed in the MDA-MB-231 cell line [260]. This same study showed that the

sFRP1 expressing cells struggled to form tumors upon inoculation into the mammary fat pads of mice and their propensity to metastasize to lung was greatly impaired [260].

Specific to the maintenance of CSCs, Wnt/β-catenin signaling is implicated in many cancers [223, 224, 261-268], including breast cancer [269]. For example, radiation resistance of mouse mammary stem/progenitor cells has been correlated with overexpression of β-catenin in the stem cell survival pathway [266]. Additionally, overexpression of Wnt/β-catenin signaling was reported to promote expansion of the hepatic progenitor cell population in animal studies [267] and the elimination of β-catenin abrogates chemoresistant cell populations endowed with progenitor-like features [57]. Of great interest is the link between Wnt/β-catenin and PI3K (phosphoinositide 3 kinase) /Akt (protein kinase B) pathway as established by several studies. Korkaya *et al*. demonstrated that PI3K/Akt pathway is important in regulating the mammary stem/progenitor cells by promoting β-catenin downstream events through phosphorylation of GSK3β [60, 189]. Other studies have revealed the ability of activated Akt, such as phospho-Akt Ser473 to phosphorylate Ser9 on GSK3β, thereby decreasing the activity of GSK3β, and potentially stabilizing β-catenin [270-272].

In summary, the proof of concept for inhibiting Wnt signaling in cancer is in place. Furthermore there is an increasing amount of evidence to support a role for Wnt signaling in breast cancer; thus, a target has been created for future studies. Specific to breast cancer, the emphasis on target development ranges from antagonizing Wnt ligand secretion or binding to promote β-catenin degradation to specifically blocking β-catenin-mediated transcriptional activity [222]. Nonetheless, as noted several times throughout this chapter, the cooperation of Wnt pathway with other signaling pathways in cancer is an important consideration. Aside from the challenge of determining the most efficacious way to inhibit Wnt related factors, possible safety concerns should be considered; another compelling reason to explore specific targets in the Wnt pathways for all breast cancer sub-types.

7.3. Hh pathway

A crucial mediator of normal tissue development, with recent indications as a regulator of tumor-related vascular formation and function [273], the Hh signaling pathway in cancer is activated by ligand independent mutations in the pathway or through Hh overexpression (ligand-dependent) [189, 274, 275]. In the absence of Hh ligands, (Sonic Hh, Desert Hh and Indian Hh), their transmembrane receptor Patched (Ptch) associates with and blocks the G-protein-coupled phosphoprotein receptor Smoothened (Smo) and is only released when secreted Hh ligands bind to Ptch [189, 276, 277]. This binding triggers the dissociation of glioma-associated (Gli) family of zinc finger transcription factors. The three Gli proteins found in vertebrates include Gli1 and Gli2 (thought to activate Hh target genes) and Gli3 (known to act primarily as a repressor) which lead to the transcription of an assortment of genes including cyclin D, cyclin E, myc and elements of EGF pathway effectors through complex interactions with Costal2 (Cos2), Fused (Fu) and Suppressor of Fu (SuFu) [276-278]. Somatic mutations which activate Hh pathway have been implicated in a variety of human malignancies[278] including basal cell carcinomas, pancreatic cancer, medulloblastomas, leukemia, gastrointestinal, lung, ovarian, breast and prostate cancers [274, 275, 279]. Both *in vitro* and mouse model

systems have demonstrated that the Hh signaling pathway plays a crucial role in regulating self-renewal of normal and malignant human mammary stem cells [51, 189]. Hh pathway inhibition has been shown to result in tumor growth inhibition mediated through the stromal microenvironment; as demonstrated in a xenograft model using a tumor and stromal cell co-injection procedure, and consistent with a paracrine signaling mechanism [280]. Although data describing the genetic alteration and the modulation of the expression pattern of Hh pathway components in mammary gland are limited, possible indications for the Hh pathway in development and maintenance of mammary cancer have been proposed [281]. However, a more significant role of Hh signaling has been revealed in prostate cancer studies, demonstrating that autocrine Hh signaling by tumor cells is a requirement for proliferation, viability and invasive behavior [282]. Additionally, the association of accelerated prostate cancer growth and progression with increased Hh signaling has been reported [283]. The Hh signaling pathway has been demonstrated as a critical pathway involved in stem cell self-renewal [276] including the essential role of Hh-Gli signaling in controlling the self-renewal behavior of human glioma CSCs and tumorigenicity [189, 284]. Known for its central role in the control of proliferation and differentiation of both embryonic stem cells and adult stem cells, aberrant activation of Hh signaling could be involved in the generation of CSCs and the development of cancer [278, 285]. In this regard, the development of Hh inhibitors may be a solution in the treatment of human cancers, including prevention of tumor progression. Essential similarities have been noted between Wnt and Hh signaling pathways [286] and their key roles in the physiological and pathological development of both embryonic and stem cells [278] [278]gives rise to the fact that crosstalk exists between the two. Signaling for both are activated by G-protein-coupled receptors [287, 288] and prevents phosphorylation-dependent proteolysis of key effectors (*Cubitus interruptus* or β-catenin) responsible for the conversion of a DNA-binding protein from a repressor to an activator of transcription [278, 289]. Considering the progression model of many cancers, specifically metastasis to bone, it is interesting to note that Wnt signaling has been reported to be downstream of Hh signaling, participating in bone development [278, 290]. Further proof proposing that Wnt signaling is downstream of Hh includes the ability of activated Gli1 to stimulate the transcription of Wnt ligands [276, 278]. It has been noted that molecules involved in Wnt signaling (i.e: GSK-3β) also play a regulatory role in Hh signaling [278, 286]. Furthermore, canonical Wnt/β-catenin signaling is required for the pathological response to oncogenic Hh signaling [278, 291].

7.4. NFκB Pathway

Along with their hallmark roles in cell survival, proliferation, inflammation and immunity, the NF-κB family of transcription factors are often constitutively expressed in breast cancer tumors [292]. Early studies on NF-κB pathway determined its key role in mammary epithelial proliferation, architecture and branching during early post-natal development [293, 294]. However, independent of its effects on mammary development, evidence exists to suggest that NF-κB regulates breast tumor progression [293, 294]. Constitutive activation of NF-κB in several breast tumor cell lines has been shown to profoundly affect the initiation and progression of breast cancer [295]. NF-κB is also required for the induction and maintenance of the EMT a process that critically controls breast cancer progression [296, 297]. Additionally, it is

evident that NF-κB mostly acts in specific breast cancer sub-types, namely estrogen receptor (ER)-ve and ErbB2+ve tumors [298, 299] and has been implicated in stem cell expansion in breast cancer studies [187].

Activation of NF-κB results in the constant nuclear localization of proteins including p50, p52, p65, cRel and RelB which subsequently up-regulate anti-apoptotic proteins causing an imbalance between normal cell growth and apoptotic cell death [300]. NF-κB-activation occurs mainly through two well characterized pathways, namely the canonical (classical) and the non-canonical (alternative). Both pathways systematically work in a similar fashion in that they are reliant on signal-induced phosphorylation and degradation of an inhibitory molecule to release and transport nuclear NF-κB proteins. However, they differ in the types of trigger signals, activated kinases, inhibitory molecules and NF-κB proteins utilized in each system. In addition to each of these aforementioned pathways, other NF-κB activating pathways exist and have been indicated in the initiation and progression of breast cancer, however we will not discuss these fully in this chapter other than in the context that they appear in the described and relevant research experiments.

Specifically, the canonical pathway involves translocation of a p50/p65 heterodimer to prompt the expression of genes intricated in cell proliferation as well as their survival, inflammatory properties and role in innate immunity [292]. This process occurs through a transforming growth factor beta activated kinase-1 (TAK1)-dependent pathway and is normally dependent on members of pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNFα) or IL-1β and other pro-inflammatory cytokines to degrade the inhibitor (IκBα) by the NF-κB essential modulator ((NEMO)/IκB kinase (IKK))γ-containing IKK complex [292]. In 2004, Biswas *et al.* published results regarding the activation of NF-κB in human breast tumors and in carcinoma cell lines indicating that the canonical pathway contributes to tumor development [298]. The resulting highlight of this experiment gave rise to activated NF-κB as a therapeutic target for distinctive subclasses of ER-ve breast cancers [298]. Specifically, the (NEMO)-binding domain (NBD) peptide (a selective inhibitor of IKK) blocked heregulin-mediated activation of NF-κB and cell proliferation while inducing apoptosis on proliferating cells substantiating the hypothesis that certain breast cancer cells rely on NF-κB for aberrant cell proliferation and simultaneously avoid apoptosis [298]. More recently, Connelly *et al*. [2011] showed, via genetic approaches, that the canonical NF-κB-activating pathway is inhibited in defined frames during polyoma middle T oncogene (PyVT) tumorigenesis and that interruption of this pathway in the mammary epithelium increases the latency of tumors and decreases tumor burden [301].

The non-canonical pathway, considered to be critical in adaptive immunity, is similar to the canonical cascade as it also relies on an IKKα heterodimer, but not on NEMO/IKKγ [302]. Prior to nuclear shuttling of 52/RelB dimers, the inhibitory molecule p100 is partially degraded through an NF-κB-inducing kinase (NIK)-dependent pathway [292]. Early studies revealed the enhanced expression of the NF-κB protein p52 in breast cancer samples giving rise to the involvement of the non-canonical pathway [303],[304]. The NF-κB protein RelB is increased in ERα-ve breast cancer cells and is required for the maintenance of mesenchymal ERα-ve breast cancer cells partially through the transcriptional induction of BCL2 [305]. Furthermore, RelB/p52 complexes have since been implicated in mammary carcinogenesis. For example, mouse

mammary tumors induced by 7,12-dimethylbenz(a)anthracene treatment have been shown to increase RelB/p52 activity and the inhibition of RelB in breast cancer cells repressed cyclin D1 and c-Myc levels and growth in soft agar [306]. Perhaps the most conclusive proof of non-canonical NF-κB-activating pathway involvement occurred in studies employing a novel transgenic mouse model to consider the role of involved mediators (downstream of p100/p52] in both mammary development and tumorigenesis [307]. The results of this study indicated an increase in p100/p52 expression in tumors from mice expressing PyVT in the mammary gland, [307] with no change of nuclear p65 detected; an indication that the observation was limited to a deregulated non-canonical NF-κB-activating pathway [307].

7.4.1. NF-κB and breast cancer stem cell renewal

Recently, studies have strengthened the association between stem cells, breast cancer and NF-κB. Such have been captured in a review by Shostak and Chariot highlighting experiments to date complemented by a compelling rationale for targeting NF-κB and other developmental pathways involved in the self-renewal of normal stem cells [187]. The involvement of NF-κB in various signaling cascades has proven critical in several studies involving breast cancer stem cell expansion.

Cao *et al*. [2007] showed that IKKα is both a regulator of mammary epithelial proliferation and a contributor to ErbB2-induced oncogenesis [308]. Specifically, breast cancer cells from IKKα (AA/AA) knock-in mice (whereby IKKα activation is disrupted) crossed with the Her2 murine breast cancer model, exhibited diminished self-renewal capacity and resulted in the inability to establish secondary tumors [308]. Breast cancers that generate primary, as opposed to secondary mammospheres such as seen in mice used in these experiments, suggests that IKKα is likewise required for the self-renewal of tumor-initiating cells from the Her2 breast cancer model [187]. Additionally, mutated IKKα slowed tumor development following exposure to 7,12-dimethylbenzaanthracene or the MMTV-c-neu (ErbB2/Her2) transgene; however there was no effect on MMTV-v-Ha-ras-induced cancer despite the fact that both of these oncogenes rely on cyclin D1 [308]. In this same series of studies, carcinoma cells from another mouse model (IKKα(AA/AA)/MMTV-c-neu) underwent premature senescence when cultured under conditions used for propagation of mammary gland stem cells. Altogether, these mouse models of breast cancer show that IKKα seems to act as a central protein in the activation of NF-κB during breast cancer stem cell self-renewal [308]. Therefore, the researchers concluded that IKKα may represent a novel and specific target for treatment of ErbB2+ve breast cancer.

While NF-κB appears to be activated in luminal progenitor cells during differentiation of mammary colony-forming cells [309], the mammary stem-like basally located cells are devoid of NF-κB activity [309, 310]. Taken together, these studies suggest that only the canonical NF-κB pathway is active in normal luminal progenitor cells before transformation and is required for the formation of mammary luminal-type epithelial neoplasias [309]; a reminder of the importance of understanding the cellular etiology underpinning breast tumor heterogeneity [310].

Another interesting role for NF-κB signaling involves the link between inflammation and cancer. While the mechanism linking inflammation and cancer has yet to be explained, we do know that the inflammatory cytokine, interleukin(IL)-6 is up-regulated in epithelial cancers, including breast cancer [311]. We also know that NF-κB regulates the expression of anti-apoptotic genes and activates different pro-inflammatory cytokines and chemokines, including IL-6 [312, 313]. Further clarity on the interactions of NF-κB signaling, inflammation and cancer has been gained through a study showing that the temporary activation of Src oncoprotein mediates an epigenetic event whereby immortalized breast cells are stably transformed to a cell lines that represent self-renewing mammospheres containing cancer stem cells [313]. The inflammatory response triggered by the activation of Src and further downstream signaling which inhibits IL-6 expression is mediated by NF-κB [313]. It has been shown that the transformation of cells utilized within this experiment occurs via a positive feedback loop whereby IL-6 mediated STAT3 transcription factor stimulation activates NF-κB [313]. These authors have demonstrated that Src activation triggers a rapid inflammatory response mediated by NF-κB that is critical for cellular transformation. While this study defines Src's role as an oncogenic kinase promoting the expansion of breast cancer stem cells, it also demonstrated the critical involvement of NF-κB in the process [313].

It is known that the onset of progestin-driven breast cancer is affected by the deletion of IKKα in mammary-gland epithelial cells [314]. Such studies are relevant in breast cancers as they consider the importance of associated risk factors between hormone replacement therapy (i.e.: progesterones or synthetic derivatives) and the increased risk of incident of fatal breast cancer [314]. The expression of both receptor activator of NF-κB (RANK) and RANK ligand (RANKL) have been observed in primary breast cancers in humans and breast cancer cell lines [315]. Studies to date indicate that the RANKL/ RANK system is mediated in part by IKK-α–NF-κB signaling and controls the incidence and onset of progestin-driven breast cancer; more specifically a loss of RANK expression significantly impairs the self-renewal capacity of cancer stem cells [314, 316]. Thus, because of the link between the RANKL/RANK system and progestin-driven epithelial carcinogenesis, RANKL inhibition could be considered as a novel approach to the prevention and/or treatment of breast cancer [314].

More recently, a model of Her2-dependent tumorigenesis indicated that breast cancer stem cell renewal is regulated by epithelial NF-κB through a reduction in the expression of key embryonic stem cell regulators, namely Sox2 and Nanog [317]. Specifically, NF-κB was required for both proliferation and colony formation of Her2-derived murine mammary tumor cell lines [317]. Additionally, the rate of initiation of Her2 tumors was governed by NF-κB [317].

7.5. Summarizing pathway interruptions/targets

In many human breast cancers, all three developmental pathways (Wnt, Notch and Hh) appear to be deregulated and control the self-renewal of normal stem cells from a molecular perspective [186]. Additionally, the involvement of NF-κB has emerged as another involved pathway in breast cancer based on what we know about Her2, a membrane bound receptor tyrosine kinase. Her 2 is overexpressed in 30% of breast cancers and critically controls the cancer stem-cell population [318]. Since Her2 activates NF-κB through the canonical pathway [319] [27],

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the hypothesis exists that the NF-κB pathway may be involved in the biology of breast cancer stem cells. It is also obvious that these interrupted pathways, and likely others unknown at this time, are responsible for certain stages of cancer progression or cancer cell aggression. If so, then we are in agreement with others who have noted that the development and identification of selective inhibitors of specific signaling pathways is an attractive approach for the prevention of tumor progression and/or treatment of cancers [278]. However, we have also mentioned several examples of cross-talk between the components of different cell signaling pathways. This concept then introduces the task of targeting multiple pathways in an effort to prevent progression and metastasis of cancers. Alternatively, and based on the premise that certain pathways operate downstream of others, it would be more reasonable to focus on inhibitors of overarching pathways, such as NF-κB. In summary, such oncogenic pathway signatures are fundamental in natural product testing. Therapeutic approaches involving natural products may provide a link between pathway deregulation and therapeutic sensitivity indicating an opportunity for the development of target compound(s).

8. Prevention and treatment of breast cancer with natural products: Past successes and promising future treatments

Currently, hormones and cytotoxic drugs remain the standard treatment for metastatic breast cancers. Development of a therapeutic approach for treating tumors, tumor reoccurrence and metastatic tumors is crucial for reducing mortality in cancer patients [6]. There is an urgent need to explore agents that will be effective in preventing and treating metastasis of breast cancer. For centuries, nature has provided us with a rich source of compounds for various disease treatments. Such naturally-derived molecules have been utilized in formal drug discovery platforms of the pharmaceutical industry. Greater than 60% of new chemical entries at the National Cancer Institute from 1981-2002 were either natural products or were derived from natural products [320]. Such naturally-occurring sources can be defined by their origin and include biotic (i.e.: forests, plants, animals, birds and marine organisms) and abiotic (i.e.: land, water, air and minerals such as gold, iron, copper, and silver) components [321]. Within these categories, plants have proven to be a rich source of lead compounds (i.e: alkaloids, morphine, cocaine) or the basis for synthetic drugs (i.e: anesthetics from cocaine) [320, 321]. The complexity and variation of plant structures indicates that their evolution has naturally completed the screening process and that the creation of potent compounds makes them more likely to survive. Plants offer the advantage of abundance, and even with such clinical successes as paclitaxel (Taxol) from the yew tree, and the antimalarial agent artemisinin from *Artemisia annua*, the vast majority have not been studied [320]. Due to promising bioactivity and diversity, the plant environment offers a potential source of natural products. A vast number of studies in the discipline of epidemiology have confirmed an association between fruit and vegetable consumption and the reduced risk of several cancers resulting in an increased interest in the role of naturally occurring dietary compounds in the efficacy of cancer chemoprevention [322]. Thus, the exploration of plant-based molecules as anti-cancer drugs is appropriate.

The history of Tamoxifen and its derivatives in the successful treatment of estrogen receptor (ER)+ve breast cancers are well documented. In the past Tamoxifen was successful in reducing breast cancer mortality rate in hormone receptor+ve breast cancer patients by up to a third and thus was the stronghold of endocrine treatment [323]. Clinical trials have indicated that aromatase inhibitors (AI) have improved efficacy compared with Tamoxifen for the treatment of post-menopausal hormone receptor+ve patients [324-327]. Additionally, the response rate for third generation AIs as first-line agents range from 30%–50% in ER+ve advanced breast cancer. [323]. Leading to these discoveries, and in an effort to capitalize on the advantages of both anti-aromatase and anti-estrogenic activity, many natural products have been tested for their ability to prevent and treat breast cancer, *in vivo* and *in vitro*. Table 4 summarizes plant based compounds that have been indicated for their potential as a prevention or treatment in breast cancer. This list is not exhaustive; however it does capture the extracts studied to date according to activity in cell lines and animal models and represents the most common types of breast cancer. In addition to those listed within Table 4, soy-based extracts, curcumin and piperine have been studied and we will discuss these in detail as the most promising plant based targets in the prevention, treatment and progression of breast cancer.

9. Promising plant based targets in the prevention, treatment and progression of breast cancer

9.1. Summarizing the individual and combined effects of the soy isoflavones, genistein and diadzein, on mammary tumor development, metastases and invasive breast cancer cells *in vivo* and *in vitro*

Genistein, daidzein and glycitein are the main isoflavones present in soybean and soy-based foods [341] [342]. Out of these, genistein is the mostly studied and dominant isoflavone of soy against breast cancer and has progressed to phase II clinical trials [343]. Soy isoflavones acting upon breast cancer cells *in vitro* and *in vivo* have been studied extensively with varying results and the clinical implications specific to breast cancer have been discussed. Soy isoflavones are structurally similar to female androgen estrogen, and thus they are also known as phytoestrogens [344] and may possibly be competing with the physiological estrogens. Genistein and daidazine (but not glycietein) possesses the ability to transactivate the estrogen receptors.

Utilizing cell based assays on MCF-7 human breast cancer cells, estrogenic agonist actions of soy isoflavones have been studied by Matsumura and co-workers whereby genistein and daidzein exert estrogen response in MCF-7 cells [345] via ER with higher affinity to ERβ1. Similarly, in hepatoma cells transfected with ER, genistein and daidazine bind to both ERα and ERβ but with more affinity to ERβ. Genistein is more potent compared to daidazine [346]. However, these phytoestrogens are 400-600 times less potent compared to 17-β estradiol [347].

In vitro, genestein is capable of identifying cells that specifically carry BRCA1 mutation and strongly inhibits the growth of BRCA1 mutant cells compared to cells expressing the wild-type BRCA1 protein [348]. The resistance shown by cells expressing wild type BRCA1 protein has been attributed to increased AKT and decreased p21 (WFA1/CIP1) protein levels [349].

Natural Product; Active Ingredient	Effect	Ref.
White button mushrooms (*Agaricus bisporous*); Conjugated linoleic acid and its derivatives	Decreased both tumor cell proliferation and tumor weight with no effect on rate of apoptosis in MCF-7aro cells and nude mice injected with MCF-7aro cells.	[324]
Taxus brevifolia (Pacific Yew) and other Taxus derivatives; Paclitaxel	Promotes tubulin polymerization and stabilization of microtubules against depolymerization.	[328] [329]
Dysoxylum binectariferum; Flavopiridol	Inhibition of MMP-2 and MMP-9 secretion of in MDA-MB-435 (parental) and 435.eB (stable transfectants) breast cancer cells.	[330]
Green Tea; *Epigallocatechin gallate*	Suppresses receptor (ERα) MBA-MB-231 breast cancer cell growth *in vitro* and *in vivo* in combination with curcumin.	[331]
Bloodroot *(Sanguinaria Canadensis);* Sanguinarine	Induced apoptosis through mediation of ROS production in MDA-MB-231 breast carcinoma cells, decrease in mitochondrial membrane potential, release of cytochrome c, activation of casp-3, and casp-9 and down regulation of Bcl-2.	[332]
Garcinia hanburyi. (Gamboge tree); Gambogic acid	Upregulation of p53 and down regulation of Bcl-2 resulting in apoptosis in MCF-7 cancer cells.	[333]
Ganoderma lucidum; Ganoderic acids	Inhibits AP-1 and NF-κB activity; inhibition of u-PA secretion from MDA-MB-231 cells.	[334]
Ginger; Acetoxychavicolacetate	Decreased cell viability in MCF-7 and MDA-MB-231 cells via casp-3-dependent increase in apoptosis.	[335]
Grapes, fruits, and root extracts of the weed Polygonum cuspidatum); Resveratrol	Suppresses NF-κB activation and cell proliferation in MCF-7 cells, reduced expression of Cox-2 and MMP-9 (with a reduced NF-κB activation).	[336]
Garcinia indica (kokum); Garcinol	Induced apoptosis in MCF-7 and MDA-MB-231 cells via caspase activation and down-regulation of NF-κB regulated genes.	[337]
Plumbago europaea (Plumbago); Plumbagin	Induced apoptosis with concomitant inactivation of Bcl-2 and the DNA binding activity of NF-κB in MCF-7aro breast cancer cells.	[338]
Rotenone; Deguelin	Arrests cells at the S phase resulting in anti-proliferative effect in MDA-MB-231 cancer cells.	[339]
Silymarin; Silibinin	Reduced PMA-induced invasion of MCF-7 cells through specific inhibition of AP-1-dependent MMP-9 expression.	[340]

Table 4. Plant Based Natural Products indicated in Breast Cancer

Cyclooxygenase-2 (COX-2) expression, which is associated breast cancer risk [350], can also be inhibited by soy isoflavones [351]. Thus, it seems that soy isoflavones are capable of curtailing breast cancer risk factors.

The influences of soy isoflavones on cell growth, cell cycle and apoptosis are all relevant to their effectiveness as chemopreventive agents for breast cancer. A number of studies have indicated the potential of genistein to inhibit proliferation of breast cancer cells in culture by causing cell cycle arrest and/or apoptosis. Genistein induces G2/M cell cycle arrest [352-354]. This effect was seen both in hormone sensitive and hormone independent cells [352]. According to Li *et al*. [2008] G2/M cell cycle arrest occurs, via stable activation of ERK1/2 pathway [354].

Demarcation on the relative importance of cell adhesion, invasion and migration for primary tumor growth verses metastatic tumour growth is not clear. However, motility, migration and adhesion are more connected to metastasis which is undoubtedly the most life-threatening aspect of breast cancer. Thus, it is crucial to identify the effects of soy isoflavones on disease metastasis.

Microarray analysis of genistein treated HCC1395 cells, a cell line derived from an early stage primary breast cancer, has indicated up-regulation of genes that inhibit invasion and down-regulation of genes that promote invasion [355]. Genistein enhances the adhesion of breast cancer cells [356, 357]. This may possibly be one method utilized by genistein to reduce metastasis.

A study by Vantyghem *et al* (2005) describes the ability of dietary genistein to affect metastasis in a post–surgical model in mice [358]. This test model mimics the clinical situation where primary tumors are surgically removed and therapeutic strategies are applied to prevent the growth of any cancer cells seeded to other locations prior to surgery. In this study, primary tumours were established by injecting human breast carcinoma cells, MDA-MB-435/HAL, into the mammary fat pad of nude mice. After 5 weeks, tumours were surgically removed and mice were maintained with a soy free diet or genistein supplemented diet. At the end of 5 weeks, a 10 fold reduction in percent lung metastasis in mice fed on a genistein supplemented diet was seen. In another study, as described by Zhang *et al.,* genistein has shown its ability to reduce the number and volume of osteolytic bone metastases in Balb/c(nu/nu) mice injected with MDA-MB-231 human breast cancer cells [359]. As there are clear indications of the ability of this compound to inhibit breast cancer metastasis, as described in the various animal studies, it would be useful to identify the associated molecular mechanisms. Although there are no conclusive findings, a number of different mechanisms have been suggested.

In studies focused on determining related mechanisms, scientists have given more attention to molecules that are overtly expressed in malignant breast tumours. For example, much attention has been invested into the actions of focal adhesion kinase (FAK), a tyrosine protein kinase. As described previously, cell motility is an integral part of metastasis and it is justifiable to investigate the components directly involve in cell motility. FAK has been designated as a regulator of cell migration and invasion [360]. Since over expression of FAK in human tumors occurs, it has been proposed as a potential therapeutic target [361]. Increased expression of FAK expression in invasive breast carcinomas is associated with an aggressive phenotype [362]. In a transgenic model of breast cancer, mammary epithelial specific disruption of FAK blocks transition of premalignant hyperplasias to carcinomas (and their subsequent metasta-

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sis) indicating direct involvement in mammary tumor progression [363]. Further, attenuation of FAK function dramatically increased apoptosis in breast cancer cells [364]. Disruption of FAK signaling by expressing the N-terminal domain FAK in human breast carcinoma cells has led to rounding, detachment and apoptosis [365]. To gain insight into the influence of genistein and daidazine in this important pathway in breast cancer, *in vitro* and *in vivo* studies have been conducted. According to an *in vitro* study, the soy isoflavones genistein, daidzein and 17β estradiol increased the number of focal adhesions and FAK activity in ERα +ve (T47D cells) as well as in ERα -ve (MDA-MB-231) breast cancer cells indicating possible involvement of novel signaling pathways and independent of estrogen receptors. Authors of this study suggested a progressive role (to metastasis) for soy isoflavones in the activation of multiple FAK regulated signaling pathways relevant to breast cancer [366], however the mechanism was not investigated. The studies of Mitra and co-workers may possibly explain the mechanism of FAK in breast cancer metastasis [367]. According to this study, reduced FAK activity or expression blocked 4T1 breast cancer cell invasion through matrigel and the blocking was associated with a 2-3 fold reduction in the expression of urokinase plasminogen activator (uPA) [367]. uPA is a serine protease that cleaves extracellulaer matrix and stimulate plasminogen to plasmin. Cancer cells are known to digest the ECM via substances like uPA and matrix metalloproteases (MMPs) as a means of invading surrounding tissue. This idea is supported by the fact that breast cancer patients with higher level of MMP-9 in tissue is associated with lymph node metastasis; thus, MMP-9 levels in serum, tumour tissue and urine are used as prognostic markers [368]. Furthermore, a study on the role of membrane-type 1 matrix metalloproteinase (MT1-MMP) *in vitro* and in SCID mice reports that the down regulation of mammary cancer cell MT1-MMP has no effect on primary tumour growth and lymph node metastasis, but reduces the occurrence of lung metastasis [369]. Interestingly, uPA secretion from mammary carcinoma cells can be influenced by genistein. This property of genistein has been shown *in vitro* and *in vivo* and the implication on tumour angiogenesis has been studied using F3II mammary carcinoma cells in culture as well as in a syngeneic mouse model. Accordingly, non-cytotoxic concentrations of genistein (0.1-50μM) significantly reduced motility in F3II mammary carcinoma cells and inhibited the secretion of uPA from cell monolayers. Once F3II cells were implanted in syngeneic mice receiving a treatment of genistein (10mg/kg/day), anti antiangiogenic effects were evident [357].

These studies indicate the effectiveness of genistein to inhibit angiogenesis and metastasis by inhibiting proteolytic substances such as uPA. In this respect, our attempt to further explore the value of soy isoflavones in modulating metastasis enabled us to review some important findings. Studies by Shao *et al*, [1998] reported that genistein inhibited the invasion of MCF-7 and MDA-MB-231 cells *in vitro* and the inhibition was characterized by down regulation of MMP-9 (matrix mettaloproteinase-9) and up regulation of TIMP-1 (tissue inhibitor of mettaloproteinase-1) [370]. The same effects were seen in nude mouse xenografts of MCF-7 and MDA-MB-231 cells [371]. Furthermore, in MDA-MB-231 xenografts, genistein inhibited tumour growth, stimulated apoptosis, regulated p21 WAF1/CIP1 expression, inhibited angiogenesis with reduced vessel density and decreased the levels of vascular endothelial growth factor and transforming growth factor β1 [370]. Further studies by the same authors reported that genistein inhibits both constitutive and epidermal growth factor stimulated

invasion in ER- human breast carcinoma cells as characterized by up regulation of TIMP-1 as well as other trypsin inhibitors like protease nexin-II (PN-II) and alpha 1-antitrypsin (alpha 1-AT) [371]. Kousidou *et al* [2005], examining normal mammary cells (MCF-12A), low invasive (ER+ve) MCF-7 cells and high invasive MDA-MB-231 (ER-ve) cells (in parallel) showed differences in the effect of genistein on highly invasive and low invasive cells. Accordingly, all cell types expressed genes of MMP-2, MMP-9, membrane-type matrix metalloproteinase (MT-1, MT-2, MT-3), MMP and TIMP-1, -2 and -3. However, once genistein was added, down regulation of all MMP genes in highly invasive cells and down regulation of many genes in low invasive MCF-7 cells was observed [372].

Based on the above findings, genistein has a role in reducing metastasis and this appears to arise from its ability to suppress uPA and MMPs thereby invading barriers with no direct effect on the capacity of cell mobility. According to the literature, expression of uPA and MMPs is regulated by NFk-B [373] [307]. Therefore it would be worthwhile to review any association between genistein and the NF-kB pathway.

9.1.1. Genistein may act via inhibition of the NF-κB pathway

The possible connection between NF-κB and breast cancer has been extensively studied [374]. Using a doxycycline-inducible new mouse model to inhibit NF-κB activity, specifically within the mammary epithelium at the time of tumor development, Connelly *et al* (2011) indicated the active contribution of NF-κB in mammary tumor progression [301]. In this model, inhibition of NF-κB activity showed an increase in tumor latency and a decrease in tumor burden [301]. Specifically, soy isoflavones inhibited the tumors by suppressing the NF-κB pathway. Furthermore genistein potentiates the activity of a number of NF-κB mediated chemotherapeutic agents by increasing apoptosis in various cancer cells, including MDA-MB-231 breast cancer cells [375]. In the same cell line, genistein induces G2/M cell cycle arrest via stable activation of ERK1/2 pathway [354],[376]. Furthermore, the MDA-MB-231 cell line has the ability to selectively block NF-κB transactivation of IL-6, a cytokine that is known for estrogen independent tumorigenesis activity [377]. Inhibition of proteosome activity by genistein in MCF-7 breast cancer cells has also been associated with NF-κB inhibition [378]. This property of genistein is particularly important for ER deficient breast cancer as constitutive NF-κB and Mitogen- and Stress- Activated Protein Kinase-1 (MAPK) /MSK activity are linked with aggressiveness and the metastasis.

It is clear that almost all of the studies that show beneficial effects of soy isoflavones utilized genistein. However, within the natural products of soy and soy food not only genistein, but daidzein and glycitein are present. A recent study testing genistein, daidazine and glycitein separately has indicated interesting results. According to this study, only genistein induced apoptosis in MCF-7 breast carcinoma cells whereas daidzein caused a slight cell-stimulating effect in the absence of E2; thus, the authors pointed toward the possible risk of breast cancer in postmenopausal women who take soy supplements [379]. This statement is important as a number of soy supplements available in the market contain high levels of daidzein [380, 381].

An animal study using nude mice and MDA-MB-435 breast cancer cells reported the individual and combined soy isoflavones exerting differential effects on metastatic cancer progression

[382]. As described in this study, daidazine increased mammary tumour growth by 38% while genistein decrease tumor growth by 33%. Moreover, the combined isoflavones increased metastasis to all the organs examined, although no effect on primary tumour growth was noted. These results have led authors to include the consumption of soy foods as a cause of increased breast cancer metastasis. Also, a number of studies by Ju *et al* [383-385] have cited enhanced growth effects of soy components in ER+ve breast cancers. However, with these studies conducted in immune compromised mice, the relevance of these findings have been criticized [386], especially as pre-treatment with genistein has shown to been protective against mammary tumors [387]. More recently, the antiproliferative activity of both genistein and quercetin has been indicated in the prevention and treatment of HER2-overexpressing breast cancer via inhibition of NFκB signaling [388]. In this study, these specific phytoestrogens inhibited proliferation in MCF-7 cell lines accompanied by an increase in intrinsic apoptotic indicators, induction of the extrinsic apoptosis pathway (up-regulating p53), a reduction in the phosphorylation level of IκBα, and negated the nuclear translocation and subsequent phosphorylation of nuclear p65 [388].

9.1.2. Sources of soy

Soybeans can be considered as the richest source of isoflavones in the human diet [389, 390], and are available in fermented and non-fermented forms. Fresh green soy beans, whole dry soybeans, whole-fat soy flour, soy milk and soymilk products such as tofu, okara and yuba are non-fermented while soy sauce, temphe, miso and natto are fermented products [391]. Additionally, products such as soy dairy substitutes, soy cheese, soy yogurt, and soy burgers seem to be popular in Western countries. The isoflavone content in various soy-based food products greatly differ. Other than soy food products, soy supplemented (categorized as a class of complementary medicine) nutraceuticals are widely consumed by Western communities. The quality and standard of these supplements are questionable. For instance, a survey carried out in the Eastern Washington Region of U.S.A. tested 13 products (7 tablet and 6 capsule formulations) by HPLC and showed that only 4 of the 13 products contained the minimum of 90% isoflavone content claimed on the label and variations in composition over time were noted [392]. Interestingly, a recent review shows that overall, most commercially available nutraceuticals are poor in quality [393].

9.1.3. Clinical studies

Although there have been a large number of studies carried out to evaluate a possible soy-breast cancer link, evidence is inconclusive. The two main theories tested involve the effect of soy isoflavone consumption in risk of breast cancer incidence and its effect on recurrence. The largest population based cohort study, including 5,042 female breast cancer survivors, shows that soy food consumption is significantly associated with decreased risk of death and recurrence [394]. Another cohort of 1,954 female breast cancer survivors, who consumed soy isoflavones at the levels comparable to the Asian population, while undergoing tamoxifen therapy showed a reduction in the risk of cancer recurrence and no interference in the efficacy of tamoxifen [395]. Further studies have indicated that soy intake prior to cancer diagnosis is

unrelated to disease-free breast cancer survival and that the association between soy protein intake and breast cancer survival does not differ according to the presence of other risk factors such as ER/PR status, tumor stage, age at diagnosis, body mass index (BMI), waist to hip ratio (WHR), or stage of menopause [396]. No variations were noted in the soy-survival association of indicated polymorphisms in ERα and ERβ indicating that soyfoods do not have an adverse effect on breast cancer survival. A recent meta-analysis by Dong and Qin (4 studies of breast cancer recurrence and 14 studies of breast cancer incidence) revealed that the consumption of soy isoflavone is inversely associated with risk of breast cancer incidence [397]. However, the protective effect is only observed among studies conducted in Asian populations, unlike those reported in Western populations [397]. One of the previous meta-analysis studies by Wu *et al* [2008] show a similar trend. Accordingly, in Asian populations a higher intake of soy isoflavones, as compared with lower intake, is associated with 29% reduction in the risk of developing breast cancer [398]. Hence, the consumption of soy food at levels similar to those consumed by Asian populations may have protective effects. However, there is evidence in the literature to show possible adverse effects of soy due to its known stimulatory effect on the premenopausal female breast as indicated by increased secretion of breast fluid, the appearance of hyperplastic epithelial cells and elevated levels of plasma estradiol [399]. Some animal studies support the idea of related disadvantages of consuming soy isoflavones. In overiectomized athymic nude mice, physiological concentrations of dietary genistein stimulates the growth of estrogen dependent MCF-7 tumors in a dose dependent manner [383, 400]. Furthermore, the same test model showed that dietary genistein reverses the inhibitory effect of tamoxifen on the growth of MCF-7 tumors [384].

9.2. The role of curcumin and piperine in breast cancer prevention and its effects on normal human breast stem cell renewal and signaling

Curcumin is a plant derived polyphenol which gives rise to the yellow colour in the spice, tumeric. This pigment is obtained from the plant *Curcuma longa* and has been noted to have power against cancer. In their review of the mechanisms of cell cycle regulation by curcumin, Gaurisankar and Das have named it as a multiple edged sword [401] because of its ability to regulate the cell cycle as well as apoptosis. Distorted cell cycle regulation and programmed cell death/apoptosis are characteristic features of cancer and curcumin has been shown to target both mechanisms. The ability of curcumin to inhibit telomerase activity [402] and to disrupt mitotic spindle structure causing [403] micronucleation in MCF-7 breast carcinoma cells has been reported. Also, curcumin is known to induce anti-proliferative activity via the decreased expression of cyclin D1 and CDK-4 in MCF-7 breast carcinoma cells [404] and can induce apoptosis through p53 dependent Bax induction [405]. Curcumin is able to disrupt breast tumor growth, but also to inhibit metastasis.

As with genestein, curcumin has been shown to mediate its anti-cancer effects via regulation of the NF-κB signaling pathway. In the nude mouse model, curcumin suppresses the paclitaxel-induced NF-κB pathway resulting in the inhibition of lung metastasis of human breast cancer [406]. The modification of NF-kB signaling eventually leads to pro-apoptotic events and perhaps inhibition of ECM breakdown. Curcumin induced apoptosis in MDA-MB-231 cells

in vitro is associated with IkB and p65 phosphorylation and hence reduced activation of NFkB [407]. This leads to reduced expression on MMPs, diminished invasion through a reconstituted basement membrane and a lower number of metastases in immunodeficient mice injected with tumor cells via intra cardiac route [407]. The high level of of MMP-3 expression noted in MDA-MB-231 invasive breast carcinoma cells is not evident on MCF-7 non-invasive breast cancer cells, implicating its importance in invasion and metastasis. The possibility of using the major forms of curcuminoids, curcumin, demethoxycurcumin, and bisdemethoxycurcumin (all of which are found in turmeric powder) as MMP-3 inhibitors to modulate MMP-3 expression has been suggested [408]. According to Chiu and Su (2009), curcumin inhibits proliferation by increasing the Bax to Bcl-2 ratio while inhibiting the migration via decreasing NF-kB p65 expression in breast cancer MDA-MB-231 cells [409]. Utilizing microarray gene expression analysis on MDA-MB-231 breast cancer cells, Bachmeier *et al.* demonstrated the ability of curcumin to downregulate inflammatory cytokines CXCL-1 and -2 via suppression of NF-κB translocation. Moreover, silencing CXCL-1 and -2 resulted in a downregulation of several metastasis promoting genes [410].

Interestingly, curcumin can interfere with estrogen-mimicking pesticides such as endosulfane, DDT and chlordane [411].

9.2.1. Sources of curcumin

Curcumin is generally considered to be the most active component and the principal curcuminoid found in tumeric [411]. The spice tumeric is commonly used in curries and contains 2-8% of this active ingredient [412], however a supplement form is also available.

9.2.2. Clinical trials

Little data is available on the pharmacokinetics and metabolism of curcumin in humans. Dose-limiting toxicity is not reported and high oral doses of curcumin (up to 12g/day) have been tested [220, 413, 414]. In a phase one clinical trial involving individuals with non-invasive cancer and pre-cancerous conditions, oral dosing of 4g, 6g and 8g of curcumin yielded peak serum concentrations of 0.51 +/- 0.11microM, 0.63 +/- 0.06 microM, and 1.77 +/- 1.87 microM, respectively. Peak serum concentrations of curcumin are seen 1-2 hours after oral intake and this gradually declines within 12 hours [220]. In another phase I clinical trial involving 15 patients with advanced colorectal cancer, 3.6g of curcumin daily for up to 4 months was well-tolerated [413]. Another study examined the pharmacokinetics of 450mg-3600mg curcumin (daily for 1 week) in twelve patients with hepatic metastatic disease from primary colorectal adenocarcinomas. Using a high-performance liquid chromatography assay, low nanomolar levels of the parent compound and its glucuronide and sulphate conjugates were found in the peripheral or portal circulation; despite its absence in liver tissue, trace levels of products of its metabolites were detected [415]

Due to the poor bioavailability of curcumin systemically, high priority has been given to study its potential against colorectal cancers. A very recent publication on a phase IIa clinical trial involving men and women 40 years of age or over and smokers that carry 8 or more colorectal

aberrant crypt foci (ACF) indicates that oral dosing of curcumin (4g per day for 30 days) significantly reduces colorectal ACF, a biomarker of colon carcinogenesis. [416] The reported anti-carcinogenic effect of curcumin is not associated with increased levels of curcumin in local tissue but increased levels of conjugate concentrations in suggesting that curcumin may mediate its effects by cuccumin conjugates delivered systemically. The same study showed that the presence of curcumin conjugates in plasma and tissue prior to treatment (believed to be originated from the normal diet of the studied population) were accompanied by a steady increase of curcumin conjugates following the month-long daily dosing [416]. A study examining the pharmacokinetics of curcumin at the concentrations of 10g and 12g in twelve healthy volunteers indicates comparable results. Accordingly, a single dose of orally administered curcumin resulted in the detection of conjugates, glucuronides and sulfates in plasma in all subjects while free curcumin was evident in only one subject [414]. Even though curcumin conjugates and other breakdown products have not been assessed for their anticarcinogenic properties [416], these findings shed some light on the potential of curcumin as a treatment of all cancers, including those of colorectal origin. This may offer a likely explanation of how continuous exposure to small quantities of curcumin via normal diets protects Asian women from breast cancer.

A phase I dose escalation trial of combined effects of docetaxel and curcumin in patients with advanced and metastatic breast cancer was published very recently. This study involved 14 patients and demonstrated the feasibility, safety and tolerability of a combination of curcumin with a standard dose of docetaxel which warrants further investigation and progression to a Phase II clinical trial [417]. Similarly, the curcumin inhibiting effects of chemotherapy induced apoptosis in models of human breast cancer have been identified [418].

9.2.3. Curcumins' ability to destroy cancer stem cells

The properties of CSC's are connected with major signaling pathways. The signaling pathways active in mammary stem cells are shown to be Wnt/β catenin, Hh and Notch [60],[51],[185], [206],[269].

A recent study by Karkarala *et al.* has demonstrated the potent inhibitory effect of curcumin and piperine on Wnt/β-catenin signaling in primary human breast epithelial cells [419]. In this study, inhibition of Wnt signaling pathway was shown to affect breast stem cell renewal by inhibiting the mammosphere formation. According to the authors, curcumin and piperine (separately and in combination) inhibited breast stem cell self-renewal; however toxicity to differentiated cells was not reported. The plasma concentration of curcumin in people taking high oral doses has been shown to be very low due to many reasons such as metabolism of the compound in the intestine and the liver, as reviewed by Burgos-Moron *et al.* [420].

The lack of bioavailability of curcumin was known as a potential disadvantage for years and various strategies have been investigated to overcome the problem. One such strategy has been the use of piperine in combination of curcumin. Accordingly, concomitant administration of piperine and curcumin tends to increase the bioavailability (up to 2000%) compared to

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administration of curcumin alone in an experimental group of people [421]. This finding could well be a possibility as piperine has been shown to inhibit P-glycoproteins and CYP3A4 expressed in enterocytes where the bioavailability of many orally ingested compounds are determined [422]. Alternatively, increasing the solubility of curcumin by heat as means of increasing the bio availability has been suggested [423]. This method is easily achievable and a well-cooked curry with tumeric and piperine could be a tasty way of obtaining the goodness of these natural compounds.

Based on the ability of curcumin and piperine to inhibit CSC's as described above, curcumin has great potential as a possible therapeutic agent against breast cancer. The majority of the breast cancer patients have tumors that respond to the naturally occurring hormone, estrogen. Therefore, most of the currently available drugs known to be effective against breast cancer can prevent the action of estrogen and are thus referred to as selective estrogen receptor modulators (SERM). Unfortunately, there is a cohort of patients whose tumours do not express estrogen receptor. SERMs are of no use for this group with ER -ve breast cancers. The potential of curcumin and piperine to suppress the self-renewal of stem cells could prove beneficial in ER +ve as well as ER -ve breast cancer patients.

10. Summary

Considering the existence of CSC's in breast cancer, and the inability of current therapeutic approaches to destroy such, we propose targeting CSC's as a tool to investigate the effect of natural dietary compounds. Specifically, we suggest isolates of soy and turmeric, and their effects on breast cancer tumors and metastasis reoccurrence in breast cancer. Similar to other developmental agents aimed at cancer signaling pathways, the optimal dosing, dosing regimens and adverse effects will have to be refined. However, these prospects are notably different than conventional therapeutics in several ways and such should be contemplated upon exploring such molecules. For example, consider the complex cross-talk of both inter- and intra-cell signaling pathways and how feedback mechanism effects may eliminate inhibitors via the actions of a single pathway. Figure 2 summarizes targeted areas of interruption via the major pathways involved in stem cell renewal as well as those pathways that may be responsible for downstream signaling to these major pathways. Additionally, if CSC's are primary targets, then metastasis incidence and/or cancer free survival may be a more appropriate efficacy endpoint in clinical trials than tumor volume. Perhaps then it would be more strategic in breast cancer to design preclinical trials based on incorporating combination regimens at the early stages of drug development while targeting multiple pathways and focusing on appropriate endpoints; all to avoid missing potentially important therapeutic benefits. While plant based derivatives, such as those considered in this chapter hold promise in breast cancer treatment and management, their development should be pursued systematically, guided by sound scientific principles.

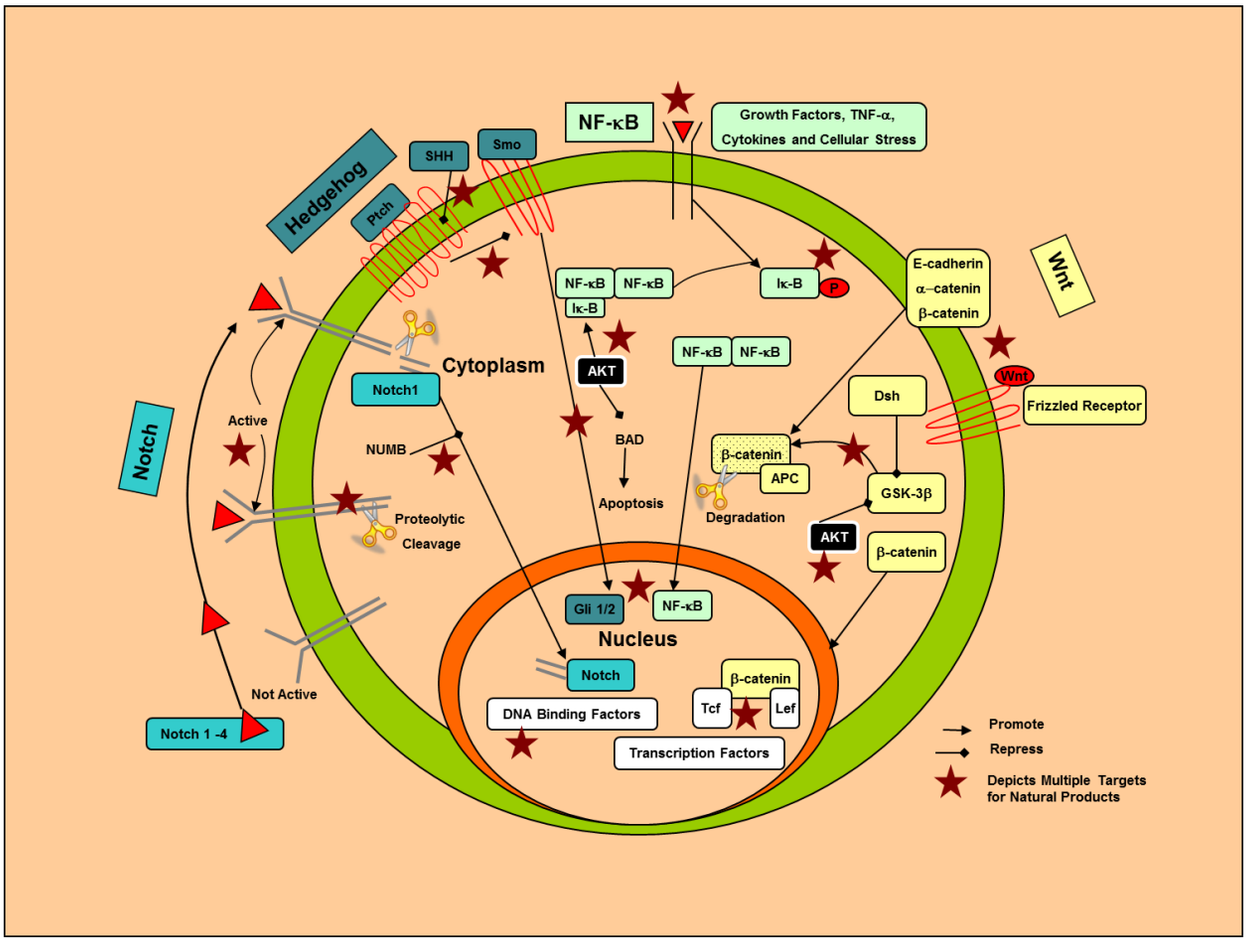

Figure 2. (Tobin, GA, 2012) An Overview of the Major Signaling Networks Involved in Breast Cancer and CSC Self-renewal, including Notch, NFκB, Wnt and Hh. Symbols and acronyms have been discussed previously, with the exception of P = phosphorylation, BAD = Bcl-2-associated death promoter, and SHH = Sonic Hh. Also, NUMB references the Protein numb homolog that in humans is encoded by the NUMB gene.

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