Notch Signaling Is Important in the Survival, Proliferation, and Self-Renewal of the Putative Breast Cancer Stem Cell Population

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NOTCH SIGNALING IS IMPORTANT IN THE SURVIVAL,
PROLIFERATION, AND SELF-RENEWAL OF
THE PUTATIVE BREAST CANCER STEM CELL POPULATION

A DISSERTATION SUBMITTED TO
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IN CANDIDACY FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

PROGRAM IN MOLECULAR AND CELLULAR BIOCHEMISTRY

BY
PETER GRUDZIEN

CHICAGO, IL
DECEMBER 2010
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<td>ABC</td>
<td>ATP binding cassette</td>
</tr>
<tr>
<td>ADAM</td>
<td>a disintegrin and metalloprotease</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>AF (-1,-2)</td>
<td>activation function (domain) -1, -2</td>
</tr>
<tr>
<td>ALDH</td>
<td>aldehyde dehydrogenase</td>
</tr>
<tr>
<td>ANK</td>
<td>ankyrin repeats</td>
</tr>
<tr>
<td>Apaf-1</td>
<td>apoptotic protease activating factor 1</td>
</tr>
<tr>
<td>APC</td>
<td>allophycocyanin</td>
</tr>
<tr>
<td>APC-Cy7</td>
<td>allophycocyanin-cyanine 7</td>
</tr>
<tr>
<td>APO</td>
<td>apoptosis antigen ligand</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BRCA</td>
<td>breast cancer susceptibility gene</td>
</tr>
<tr>
<td>CALLA</td>
<td>common acute lymphoblastic leukemia antigen</td>
</tr>
<tr>
<td>CARD</td>
<td>caspase recruitment domain</td>
</tr>
<tr>
<td>CIR</td>
<td>CBF-1 interacting co-repressor</td>
</tr>
<tr>
<td>CK</td>
<td>cytokeratin</td>
</tr>
<tr>
<td>CR</td>
<td>cysteine rich region</td>
</tr>
<tr>
<td>CSC</td>
<td>cancer stem cell</td>
</tr>
<tr>
<td>DCIS</td>
<td>ductal carcinoma in situ</td>
</tr>
<tr>
<td>DCV</td>
<td>(Vybrant) DyeCycle Violet Dye</td>
</tr>
<tr>
<td>DISC</td>
<td>death inducing signaling complex</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagles Media</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DR-4-5,-6</td>
<td>death receptor, -4, -5, -6</td>
</tr>
<tr>
<td>DSL</td>
<td>Delta, Serrate, LAG-2</td>
</tr>
<tr>
<td>EGFR</td>
<td>epithelial growth factor receptor</td>
</tr>
<tr>
<td>EMT</td>
<td>epithelial to mesenchymal transition</td>
</tr>
<tr>
<td>ER</td>
<td>estrogen receptor</td>
</tr>
<tr>
<td>ERBB2/HER2</td>
<td>avian erythroblastosis oncogene B/human epidermal growth factor receptor</td>
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<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase 2</td>
</tr>
<tr>
<td>ESA</td>
<td>epithelial specific antigen</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence activated cell sorting</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FADD</td>
<td>Fas-associated via death domain</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FLIP</td>
<td>FLICE like inhibitory protein</td>
</tr>
<tr>
<td>GCN5/PCAF</td>
<td>p300/CBP-associated factor</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GSI</td>
<td>gamma-secretase inhibitor</td>
</tr>
<tr>
<td>HDAC</td>
<td>histone deacetylase</td>
</tr>
<tr>
<td>HES</td>
<td>hairy/enhancer of split</td>
</tr>
<tr>
<td>HEY</td>
<td>hairy/enhancer of split related with YRPW motif</td>
</tr>
<tr>
<td>HPF</td>
<td>high-powered field</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------------------------</td>
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<tr>
<td>IAP</td>
<td>inhibitor of apoptosis</td>
</tr>
<tr>
<td>IMEM</td>
<td>Improved Modified Eagles Media</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus Kinase</td>
</tr>
<tr>
<td>JIP-1</td>
<td>JNK interacting protein (-1)</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun-N-terminal kinase</td>
</tr>
<tr>
<td>LBD</td>
<td>ligand binding domain</td>
</tr>
<tr>
<td>LCIS</td>
<td>lobular carcinoma in situ</td>
</tr>
<tr>
<td>LLNle</td>
<td>cbz-leucine-leucine-norleucine-CHO</td>
</tr>
<tr>
<td>LN</td>
<td>cysteine rich Notch/LIN12 repeats</td>
</tr>
<tr>
<td>MAML</td>
<td>mastermind-like</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen activation protein kinase</td>
</tr>
<tr>
<td>MEKK</td>
<td>MAPK/ERK Kinase Kinase</td>
</tr>
<tr>
<td>MKK7</td>
<td>mitogen activated protein kinase kinase 7</td>
</tr>
<tr>
<td>MLK</td>
<td>mixed lineage kinase</td>
</tr>
<tr>
<td>MMTV</td>
<td>mouse mammary tumor virus</td>
</tr>
<tr>
<td>MKP</td>
<td>MAPK phosphatase</td>
</tr>
<tr>
<td>mTOR</td>
<td>target of rapamycin</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor kappa B</td>
</tr>
<tr>
<td>NLS</td>
<td>nuclear localization signal</td>
</tr>
<tr>
<td>NOD/SCID</td>
<td>non-diabetic diabetic/severe combined immunodeficient</td>
</tr>
<tr>
<td>PARP</td>
<td>poly (ADP-ribose polymerase)</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCNA</td>
<td>proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>PE</td>
<td>phycoerythrin</td>
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xv
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>PEST</td>
<td>proline, glutamate, serine, threonine</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphoinositol 3-kinase</td>
</tr>
<tr>
<td>PR</td>
<td>progesterone receptor</td>
</tr>
<tr>
<td>RAIDD</td>
<td>RIP-associated ICH1/ced3 homologous protein with death domain</td>
</tr>
<tr>
<td>RANKL</td>
<td>receptor activated by NF-κB ligand</td>
</tr>
<tr>
<td>RIPK2</td>
<td>receptor interacting serine-threonine kinase 2</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RPMI-1640</td>
<td>Roswell Park Memorial Institute (-1640)</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse-transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>RT-qPCR</td>
<td>reverse transcriptase quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>SCA-1</td>
<td>stem cell antigen</td>
</tr>
<tr>
<td>SERM</td>
<td>selective estrogen receptor modulators</td>
</tr>
<tr>
<td>SODD</td>
<td>silencer of death domains</td>
</tr>
<tr>
<td>SRC</td>
<td>steroid receptor co-activator</td>
</tr>
<tr>
<td>SHARP</td>
<td>SMRT/HDAC associated repressor protein</td>
</tr>
<tr>
<td>SKIP</td>
<td>Ski-interacting protein</td>
</tr>
<tr>
<td>Smac</td>
<td>second mitochondrial derived activator caspase protein</td>
</tr>
<tr>
<td>SMRT</td>
<td>silencing mediator of retinoid and thyroid hormone receptor</td>
</tr>
<tr>
<td>SP</td>
<td>side population</td>
</tr>
<tr>
<td>TAD</td>
<td>transactivation domain</td>
</tr>
<tr>
<td>TGF-β</td>
<td>transforming growth factor-beta</td>
</tr>
<tr>
<td>TNFα</td>
<td>tumor necrosis factor alpha</td>
</tr>
<tr>
<td>TNF-R1</td>
<td>tumor necrosis factor receptor</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Name</td>
</tr>
<tr>
<td>---------</td>
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</tr>
<tr>
<td>TNFRSF</td>
<td>tumor necrosis factor receptor super family</td>
</tr>
<tr>
<td>TRAIL</td>
<td>TNF-related apoptosis inducing ligand</td>
</tr>
<tr>
<td>TRADD</td>
<td>TNFRSF1A-associated via death domain</td>
</tr>
<tr>
<td>TRAF</td>
<td>TNF-receptor associated factor</td>
</tr>
<tr>
<td>XIAP</td>
<td>X-inhibitor of apoptosis</td>
</tr>
</tbody>
</table>
ABSTRACT

Purpose: Numerous studies have identified stem-like cells, termed cancer stem cells (CSCs), in breast tumors and established cell lines. It has been hypothesized that CSCs are responsible for breast cancer formation, progression and recurrence; therefore, a deeper understanding of the signaling pathways regulating CSC survival will benefit development of novel therapeutic strategies. Notch signaling, which is dysregulated in breast cancer and has been implicated in mammary stem cell self-renewal, and can be effectively blocked by gamma-secretase inhibitors (GSIs). While GSIs are currently in clinical trials for breast cancer, it is not fully understood how these compounds will affect CSCs or if CSCs from different breast cancer phenotypes (estrogen receptor-alpha (ER\(\alpha\))-positive, ER\(\alpha\)-negative, Her2/neu overexpressing) will be differentially affected.

Experimental Design: The stem cell-like population was isolated using several different methods including side population, mammosphere formation and aldehyde dehydrogenase activity (Aldefluor). Notch pathway activity was analyzed in CSCs derived from cell lines and primary tumors of different breast cancer phenotypes using quantitative reverse transcriptase-polymerase chain reaction (RT-qPCR) and confirmed by Western blotting. The effect of Notch inhibition on secondary mammosphere formation and colony formation in soft agar was evaluated. Furthermore, apoptosis was confirmed after treatment with one GSI, MRK003, by TUNEL assay and Annexin V - propidum iodide staining. The mechanism of apoptosis was explored utilizing a RT-qPCR based approach as well as several kinase inhibitors including those that inhibit c-Jun N-terminal kinase and the p38 mitogen activated kinase.
Results: The CSCs possessed elevated levels of Notch activation compared to the non-CSCs regardless of breast cancer phenotype, sample origin (cell culture or primary tumor) or method of isolation / enrichment. Blockade of Notch signaling with three structurally distinct GSIs, a specific Notch decoy protein or an siRNA targeting Notch-1 abolished secondary mammosphere formation and/or blocked colony formation in soft agar and decreased the side-population.

Conclusions: These findings support a role for Notch signaling in CSC self-renewal and proliferation, and they suggest Notch inhibition may have clinical benefits in targeting CSCs. Furthermore, our data shows that Notch may be important in the survival of mammospheres and the c-Jun N terminal kinase and/or p38 MAPK pathway may play a role in MRK003- induced apoptosis.
Normal Development of the Breast

The mammary epithelia consists of a network of ducts which branch and invade the mammary fat pad before birth (1-6). During puberty the ducts start to branch due to hormone stimulation. At this phase, rapid growth is observed in the ducts. In some places, the ducts will stop growing and form structures called terminal end buds. After puberty, these buds will differentiate to form alveolar buds (this requires hormonal stimulation) (7). With pregnancy and lactation, lobulo-acinar structures form; these contain alveolar cells responsible for the secretion of milk. Then at weaning, apoptosis occurs, and the gland returns to its former, pre-pregnancy structure (3).

There are two different types of cells in the mammary gland: luminal cells, which line the ducts and lobules and myoepithelial cells, which line the basement membrane. Several markers are used to identify the luminal cells including cytokeratins (CK) -5, -7, -18, -19, the estrogen receptor (ER), and the progesterone receptor (PR). Myoepithelial cells are characterized by a different set of markers including laminin, CD10, and CK-5, -14, and -17; they do not express the ER (8). Figure 1 shows the structure of the terminal end bud along with the different cell types discussed.
The development of the mammary gland is regulated by hormone production and by small peptides produced by either stromal or epithelial cells including: Receptor Activated by Nuclear Factor-Kappa B (NF-κB) ligand (RANKL, osteoprotegerin ligand) (9), members of the Hedgehog family (10), and Transforming Growth Factor-beta (TGF-β) families (11). In the breast, the absence of RANKL causes a lack of proliferation and differentiation of the alveolar buds even though the mammary gland forms normally up to that point. This means lactation is not possible in the absence of RANKL. To illustrate this point, pregnant RANKL−/− mice were unable to lactate until RANKL was implanted. TGF-β3 has been shown to be involved in apoptosis that occurs immediately after weaning. Specifically, after milk production ceases, TGF-β3 is significantly upregulated in the mammary epithelium, which precedes apoptosis (11). The upregulation is due to milk stasis, not hormonal changes. Once initiated, apoptosis occurs through the alteration of cell cycle genes such as c-jun, c-fos, c-myc, and caspases, as well as, Bax and Bcl-Xs (apoptosis promoting factors) (11). The Hedgehog pathway is also involved in many developmental processes including skin, limbs, lung, eye, and nervous system among others. The ligand Hedgehog binds to the receptor Patched, which then activates the downstream target Smoothened that is responsible for activation of the Gli family of transcription factors (Gli1, Gli2, and Gli3) leading to target gene expression. Lewis et al. determined that a haploinsufficiency at the Patched locus leads to defects in ductal structures, as well as, minor changes in the terminal end buds in heterozygous post-pubescent virgin animals. These defects are reverted during lactation but return during gland development (10).
Estrogen and the Estrogen Receptor

Estrogen and the ER play an important role in normal breast development. Both are required for proliferation during the menstrual cycle and pregnancy (12). The ER is a steroid hormone receptor that belongs to a family of nuclear receptors (13). It is mainly found in luminal epithelial cells located in the lobules and is not found in cells expressing the basal marker called common acute leukemia lymphoblastic antigen (CALLA) (14). There are two isoforms of the receptor: ERα and ERβ. ERα has been extensively studied and is made up of several different domains, one of which is the ligand-binding domain (which contains the activation function-2 domain (AF-2), (Figure 2). The ligand 17-β estradiol (also referred to as estradiol) is a lipophilic molecule that enters the cell through the membrane and binds to the ligand-binding domain of the ER. This allows the
ER to dimerize and bind to specific estrogen response elements in the promoters of various genes. Estradiol plays a role in the growth and differentiation of the female reproductive organs (15). Specifically, estrogens will regulate growth and differentiation of various tissues in the body (16). When estradiol binds to the receptor a conformational change occurs whereby helix 12 of ERα binds to helices 3, 5/6 and 11 allowing for the activation of the ligand-binding domain. Once the ligand binds to the receptor several transcriptional co-activator proteins including: steroid receptor co-activators (SRC-1) and GRIP1 (SRC-2) will bind to the ligand-binding domain (17, 18). In general, estradiol induces expression of genes involved in cell cycle regulation including cyclin A, cyclin D1 and survivin; and downregulates genes associated with apoptosis such as caspase 2 and caspase 9 among others (19). Another domain, AF-1, is regulated by growth factors, which use the mitogen activated protein kinase (MAPK) pathways (20).

ERβ was originally cloned by Kuiper et al. in 1996 (21). Its structure is similar to ERα, but the ligand-binding domain has only 59% homology with ERα. The AF-1 regions are quite dissimilar with 16% homology, but the DNA binding domain had 97% homology between the two receptors (21, 22) (Figure 2). The role of ERβ in breast development is unclear. One study suggests that ERβ may repress ERα function, which would make ERα insensitive to hormones during lactation (23). With respect to cancer, another study showed that ERβ mRNA levels were decreased in invasive carcinomas compared with the normal mammary tissue (24). Recent evidence suggests that ERβ may have a protective role against breast cancer development. In fact, in many breast tumors ERβ has been lost, probably due to methylation of its promoter, which is common in cancer. This suggests ERβ may be a tumor suppressor (reviewed in (25)).
The Progesterone Receptor

The progesterone receptor (PR) is also important in breast development and is believed to be responsible for the formation of the lobo-alveolar structures during pregnancy. It is also likely responsible for the proliferation and differentiation of cells during lactation. PR activation is very similar to that of the ER. It involves binding of progesterone, dimerization of the receptor, binding to the progesterone response element and transcription of various genes including cyclin D1, cyclin dependant kinases, c-fos, and c-myc (26).

Stem Cells in Normal Breast Development

Stem cells are important in the development of the mammary gland, and it is believed that the terminal end buds contain stem cell activity. These cells, called cap cells, will elongate the terminal end buds and give rise to the myoepithelial layer, while cells called “body cells” will give rise to the luminal epithelial cells. These cells will
undergo apoptosis and give rise to the lumen. Cap cells also have the ability to migrate into the lumen (Figure 1) (reviewed in (2)). After pregnancy and weaning, the mammary gland reverts to the pre-pregnancy state via apoptosis. Therefore, in subsequent pregnancies, a source of new cells must be present in the normal mammary gland to reform the alveolar structures needed for lactation. Mammary stem cells fill this role (3).

Several studies have been done in mice that support the presence of stem cells in the mammary gland. One study by Daniel et al. used transplantation of small epithelial fragments into the mammary fat pad of mice (27). They demonstrated that a fully functional mammary gland could be generated from these fragments. They also showed that these fragments could be serially transplanted up to four times to form a functional mammary gland (27). Next, Kordon and Smith used mammary epithelium marked with mouse mammary tumor virus (MMTV) and showed that clonally dominant populations were responsible for generating a fully functional mammary gland (28). Also, in 2002, Welm et al. used BrdU labeling, immunosorting and transplantation to identify a population of stem cells that could regenerate the mammary gland (29). These cells were positive for the murine stem-cell antigen SCA-1, and could exclude a nuclear dye, Hoescht, a characteristic of stem cells (30, 31). Finally, in 2006, Shackleton et al. found that a mammary gland could be generated in mice from a single stem cell (identified by markers Lin⁻ CD29⁺ CD24⁺) (32). They could then re-isolate the stem cells from the newly formed mammary gland, implant them into naïve mice, and form another functional mammary gland.

Dontu et al. proposed a theory on the origins of ERα-positive and negative cells in normal human breast development. In normal breast development, an ERα-negative
stem cell generates both myoepithelial and ductal epithelial progenitor cells, which is consistent with the observation that estrogen stimulation is not required for early mammary gland development (33). The ERα-negative stem cell can differentiate into either ERα-negative or ERα-positive progenitor cells; ERα-positive progenitors will proliferate in response to estrogen later in development. When estrogen levels decrease, proliferation will cease. ERα-positive progenitor cells can form more differentiated ERα-positive or ERα-negative cells. Some evidence that supports this theory includes the fact that ERα-positive cells were not found in utero in the human mammary gland until about 30 weeks. In ERα-knockout mice there was no ductal or lobo-alveolar growth observed and terminal end buds did not form (33).

Breast Cancer Relapse and Resistance

Breast cancer is one of the leading causes of death in women with about 40,000 deaths each year in the USA alone (34). A large part of mortality is due to the development of multidrug resistance by cancer cells, which hinders chemotherapy (35). When treatment is started, chemotherapeutic drugs are active in about 90% of patients with primary breast cancer, while they are active in only 50% of patients with metastatic breast cancer. However, after some time patients develop resistance to their treatments and the cancer will likely recur (36). “Patients with recurrent breast cancer usually die of their disease” (37). Recurrence happens in about 30% of patients that have early breast cancer (36). Survival after recurrence is based on several factors including where the recurrence is localized. Poor survival is associated with recurrence in soft tissue, bone and viscera (37). When relapse occurs, the resulting cancer is often more drug resistant (36). Therefore, it is imperative to explore the mechanisms of relapse in order to identify
Breast Cancer Classification

The conventional classification of breast cancer includes early breast cancer, which accounts for about 85% of cases, locally advanced cancer (10%), and metastatic breast cancer (5%). Locally advanced cancer includes inflammatory breast cancer, a rare type of cancer (~3%) which will be discussed in detail later (reviewed in (38)). Breast cancer can also be classified by histology. An example of histological characterization includes either ductal or lobular carcinoma. Ductal carcinoma is characterized by formation of glandular structures while lobular carcinoma is more invasive and infiltrates regions beyond the basement membrane. Yet another classification identifies breast cancers based on genetic mutations. The most common breast cancer germline mutation is in BRCA (breast cancer susceptibility gene) -1 and -2. Finally, molecular classification includes the steroid receptors found on breast cancer cells or the amplification of proto-oncogenes such as HER2/neu, as well as, the expression of genes associated with luminal-like, or basal-like cells. This classification is based on a genomic-proteomic approach that was pioneered by the group of Perou and colleagues (6).

Initially, the authors developed an intrinsic gene set which was based on paired samples of tumors. Tumor samples were obtained before and after 42 patients underwent a 15-week treatment with doxorubicin (a chemotherapeutic agent). These two samples were called paired sets. All samples were compared using a microarray with approximately 8,000 genes. They noticed that the two samples obtained for each tumor...
clustered together. This indicated that there were high levels of heterogeneity among different tumors. A set of genes was chosen based on the expression patterns of genes that varied among different tumors as compared to the paired sets (39).

The molecular classifications were defined by microarray studies using 78 breast carcinomas and a set of 456 cDNAs, which were chosen from the intrinsic gene set (6). Based on their analysis Sorlie et al. defined five different types of gene-expression based molecular classes including, luminal-like A, B/C, basal-like, normal-like and HER2-positive (6). Recently, a new molecular subtype of breast cancer has been identified which involves expression of claudins (40). Claudins are proteins that form the tight junctions between adjacent cells and are responsible for blocking the diffusion of solutes between cells as well as maintaining apical and basolateral membrane domains (reviewed in (41)). Aberrant expression of claudins has been associated with a variety of diseases including metastasis in cancer (41). These different types of cancer will be discussed in detail below along with their ER status, possible treatments and survival statistics.

**Molecular Classification of Breast Cancer**

**Luminal-like Breast Cancer**

*Estrogen receptor and breast cancer*

The luminal group is subdivided into luminal A, B and C groups. Luminal type A cancers are mainly ERα-positive as are some luminal B and C type cancers. The luminal group is identified by the expression of cytokeratin markers 8 and 18. Luminal A tumors also expressed high levels of estrogen-induced genes such as GATA3 and LIV1. Luminal B tumors have moderate expression of ERα, as well as, genes associated with
proliferation such as Ki67 and the proliferating cell nuclear antigen (PCNA). While the luminal C group has low levels of ERα expression and high levels of genes associated with the basal subtype. Because the status of the ERα is important in determining treatment of breast cancer, its role in breast cancer will be discussed next.

“Estrogen promotes cancer cell growth of the breast and uterus” (15). ERα is found in 50-80% of tumors. The presence of ERα implies lower proliferation rates and increased rates of tumor differentiation. If ERα-positive cancer is treated in the earlier stages, there is a lower rate of recurrence, but if it has progressed, there is a higher rate of recurrence. The current hypothesis on how estrogens contribute to breast cancer development states that they induce estrogen-regulated proteins that act as growth factors (reviewed in (22)). The presence or absence of ERα drives the selection of appropriate therapeutics. For ERα-positive patients, endocrine therapies are commonly used. Tamoxifen is one of a group of agents known as selective estrogen receptor modulators (SERMS) and is a triphenyl ethylene derivative that blocks ERα activation. Tamoxifen binds to the ligand-binding domain in ERα, alters receptor conformation and prevents DNA binding and transcriptional activation (reviewed in (42)). More specifically, when tamoxifen binds to the ligand-binding domain, it causes helix 12 of ERα to obscure the ligand-binding domain so that co-activators cannot bind to this region (reviewed in (15)). While tamoxifen acts like an anti-estrogen in breast tissue, it acts as an estrogen in other tissues, such as the uterus, bone and brain. This is problematic because treatment of breast cancer with tamoxifen causes abnormal cell proliferation in the uterus potentially leading to endometrial cancer. In contrast, Fulvestrant is a pure anti-estrogen. Fulvestrant binds to ERα preventing proper folding, which targets the receptor for degradation by the proteosome (reviewed in (42)).
Another class of drugs, aromatase inhibitors, are also useful for treatment of ERα-positive breast cancers. Aromatase is a cytochrome P-450 enzyme that is found in normal breast and breast cancer tissue among others. Its main function is to produce estrogens in the ovary from substrates such as testosterone or cholesterol. Non-steroidal drugs, including letrozole and anastrozole, function by blocking the aromatase enzyme, which is required for the formation of estrogen (reviewed in (43)). They bind to the heme iron in cytochrome P-450 via their nitrogen atom and prevent steroidal hydroxylation. Both letrozole and anastrozole have shown promising results in treating breast cancer in post-menopausal women, and in some studies, they have been shown to be more effective than tamoxifen (reviewed in (44)). Currently, letrozole is the standard of care for ERα-positive breast cancer. Table 1 summarizes some of the hormonal therapies that are used to treat breast cancer.

**Estrogen receptor negative breast cancer and treatment**

In the case of ERα-negative cancer such as some of the cancers in the luminal B and C group (as well as basal cancers discussed below), endocrine therapies cannot be used. Therefore, chemotherapeutic agents need to be used. Chemotherapeutic agents can be classified into several groups: taxanes, vinca alkaloids, alkylating agents, anthracyclines, antimetabolites, as well as ATP competitive inhibitors and monoclonal antibodies. Taxanes are a group of drugs that work by binding to sites of tubulin dimers and stabilizing microtubules thereby preventing mitosis. As an example, Paclitaxel, works by stabilizing microtubules, preventing cells from passing through the G₂/M phase of the cell cycle (45). Vinca alkaloids are drugs that disrupt microtubule formation preventing cell division. Alkylating agents bind to DNA and form cross-links between
DNA strands, which prevents DNA replication. Anthracyclines act as topoisomerase II poisons and intercalate between DNA strands preventing DNA replication. Finally, antimetabolites prevent purine and pyrimidine synthesis (reviewed in (46)). Other agents such as monoclonal antibodies will target the specific receptor and prevent activation. ATP competitive inhibitors will prevent the binding of ATP to a receptor, which will prevent autophosphorylation and activation of the receptor. Table 1 summarizes different chemotherapeutic agents that are discussed in this dissertation along with their targets and the types of breast cancers that they are used to treat. In some cases, chemotherapeutics can be used to treat several different types of breast cancer even though they may be discussed under only one section in the text. Importantly, some taxanes and anthracyclines, such as Paclitaxel and doxorubicin, can also be used to treat ERα-positive and ERα-negative breast cancer if the disease has become more advanced (47). The table contains only representative treatments and is not comprehensive.

In terms of survival and outcome with respect to the different types of luminal breast cancer, luminal A cancers are usually less aggressive than luminal B and C. Relapse may also be higher in the luminal B or C group. Studies have shown that recurrence of ERα-positive tumors is usually to bone; whereas, ERα-negative cancers tend to recur at visceral sites. Disease free survival was also highest in the luminal A group (5). The luminal C group had the worst outcome within the luminal group. The reason may be that luminal C cancers express some genes associated with the basal-like group (6). Whether cancers are ERα-positive or ERα-negative also has an impact on their survival after recurrence. For example, if an ERα-positive patient has visceral
recurrence, the median survival time is 16 months, but this is reduced to 10 months for patients with ERα-negative cancer (37).

**HER2-positive breast cancer**

HER2 overexpression (HER2-positive) is mainly found in basal type cancers. HER2 belongs to a family of receptor tyrosine kinases, which also includes HER1 (epithelial growth factor receptor, EGFR), HER3 and HER4. HER3 is a kinase dead receptor and HER2 has no known ligand. Upon ligand binding, HER1, HER3 or HER4 will either homo- or heterodimerize (with each other or HER2) leading to phosphorylation of a tyrosine within the receptor cytoplasmic domain, which leads downstream signaling (via MAPK for example). Downstream targets include those that control cell growth, differentiation, motility, and adhesion (reviewed in (48)). Interestingly, HER2 can homodimerize without a ligand since it is constitutively active (49). Amplification of the HER2 gene is evident in about 20% of breast cancer (50). This overexpression leads to activation of the phosphoinositol 3-kinase (PI3K) pathway, which prevents apoptosis, and when combined with a loss of p53, which occurs in 40% of breast cancers, uncontrolled proliferation may occur (51) (reviewed in (52)). The activation of HER2 leads to secretion of matrix metalloproteases that promote cell invasion and metastasis (53). Matrix metalloproteinases degrade the basement membrane and allow cells to metastasize. More specifically, HER family members can interact with focal adhesion kinase, which activates the PI3K pathway (54). Then second messengers such as inositol-triphosphate, diacylglycerol and calcium are recruited and reorganization of the actin cytoskeleton occurs which leads to tumor cell migration (55).
The most common therapeutic agent used to treat patients with HER2 overexpression is a humanized monoclonal antibody called trastuzumab (Herceptin). One possible mechanism of action for trastuzumab for killing tumor cells involves antibody dependant cell cytotoxicity. Once the antibody binds to the HER2 receptor, natural killer cells can detect the Fc portion and are recruited to the cell in order to lyse it. Evidence has shown that FcRγ⁺/⁺ mice implanted with breast cancer cells then treated with trastuzumab resulted in 96% tumor reduction (56). However, when mice that had the FcRγ deleted (FcRγ⁻/⁻) were treated with trastuzumab they had only 50% tumor reduction (56). This implies that FcRγ is important in trastuzumab initiated cell death and implicates antibody-dependant cell cytotoxicity as being partially responsible for the death of these cells. Studies showed that trastuzumab given before or after standard chemotherapy was effective in patients with HER2 overexpression (57, 58). Similar results were seen in NIH3T3 cell lines with enforced HER2 expression, where trastuzumab resulted in downregulation of the HER2 receptor (59).

Sorlie et al. did an analysis of patient survival, and relapse-free survival, and found that patients with the HER2-positive subtype had one of the shortest overall survival times among the five different breast cancer classifications (6). In addition, disease free survival was also much shorter compared to the luminal group (6). In one study addition of trastuzumab to standard chemotherapy resulted in a 52% reduction in recurrence and a 33% reduction in death (34).
Basal-like Breast Cancer

Normal-like breast cancer

Briefly, normal-like breast cancer is mainly ERα-positive and HER-2-negative (60). These cancers make up about 8% of all breast cancers and are quite aggressive (61). They also express high levels of the epithelial to mesenchymal transition (EMT) markers TWIST1 and Vimentin compared to normal breast tissue (61). Briefly, EMT is an important factor in normal development and tumor progression. It allows for epithelial cells, which are normally attached, to become mesenchymal cells so they can migrate (62). Normal-like breast tumors are characterized by genes expressed in adipose tissue and non-epithelial cells. They expressed higher levels of basal genes as opposed to luminal genes (6). In terms of prognosis, normal-like tumors had slightly worse prognosis than luminal A in patients without therapy and were responsive to endocrine therapies having a good prognosis (60).

Basal-like breast cancer

Basal tumors are derived from myoepithelial cells found near the basement membrane. Basal breast cancer occurs in about 15-25% of all breast cancer cases (reviewed in (63)). The basal-like group consists of tumors that express a basal set of genes including EGFR, CK-5, and -17 and genes in the proliferation cluster including Ki67 and PCNA.

Interestingly, there may be a link between basal-like cancers and BRCA-1 deficiency. The BRCA-1 mutation is a germ-line mutation, which is common in families that are predisposed to breast cancer. BRCA-1 is involved in DNA damage response (64), X-chromosome inactivation (65), and transcriptional regulation (66). Importantly,
BRCA-1 mutations impair homologous recombination. This may lead to double stranded break formation and activation of oncogenes. In wild-type cells, double stranded breaks are repaired by an enzyme called Poly(ADP-ribose) polymerase (PARP), which binds to single stranded breaks formed during base excision repair (reviewed in (67)). BRCA-1 deficient tumors are closely related to basal cancers based on gene array studies (5).

One possible treatment for basal tumors or BRCA-1 deficient tumors would be to inhibit PARP leading to DNA double stranded breaks that cannot be repaired by RAD51-dependant homologous recombination, leading to cell cycle arrest, and apoptosis. Farmer et al. showed that using PARP-1 inhibitors would lead to specific killing of BRCA-1/2 deficient tumors (68). Patients that lack BRCA-1/2 will have a single copy of the gene in normal tissue; however, the tumor will not have any copies leading to more DNA damage and increased PARP expression. The authors demonstrated that embryonic stem cells lacking BRCA-1 or -2 were highly sensitive to killing by PARP inhibitors compared to wild-type controls in clonogenic assays (68).

Other potential therapeutic targets for basal-like tumors include EGFR or c-Kit, a receptor tyrosine kinase, either of which can be highly expressed in basal cancer compared to normal tissue. c-Kit is responsible for growth and differentiation of cells and dysregulated c-Kit has been seen in breast cancer. Imatinib (Gleevec) is a tyrosine kinase inhibitor targeting c-Kit, as well as, the abnormal protein BCR-ABL found in chronic myeloid leukemia (69). Imatinib works as an ATP-competitive inhibitor, which prevents the autophosphorylation and activation of c-Kit (69, 70). Several clinical trials have been done with Gleevec in breast cancer. Patients with advanced or metastatic breast cancer that overexpressed c-Kit generally did not benefit from treatment with
Gleevec alone (71). Additionally, a phase II trial showed little benefit when Gleevec was combined with the chemotherapeutic capecitabine in metastatic breast cancer (72). Capecitabine is a drug which is converted to 5-fluorouracil that then binds to DNA and prevents tumor growth.

**Triple-negative breast cancer and claudin-low breast cancer.**

A specific group of basal tumors called triple negative breast cancer lack ERα, PR and HER2 receptors. Basal-type and triple negative classifications should not be used interchangeably since some basal tumors do express low levels of ER, PR or HER2 (73). Triple-negative breast cancers are identified by cytokeratins 5/6. They are very aggressive and account for 10-15% of breast cancer cases (73). Triple negative breast cancers express higher levels of EGFR and c-Kit than non-malignant breast tissue; these can be used as potential targets. EGFR can be targeted by Cetuximab and Gefitinib while c-Kit can be targeted by Imatinib as discussed. Cetuximab is a humanized monoclonal antibody that binds to EGFR and inhibits activation. Currently, Cetuximab is in clinical trials looking at its effect on triple negative metastatic breast cancer (clinical trials.gov. NCT00463788). Gefitinib, on the other hand, will block the ATP binding site on EGFR preventing its activation. The MAPK and protein kinase B (PKB/Akt) are also potential therapeutic targets in triple negative breast cancer (reviewed in (73)).

Recently, a subset of triple negative breast cancer cells has been identified bearing a claudin-low phenotype (40). Metaplastic breast cancers, which are a subset of triple negative breast cancers, are especially difficult to treat. They make up about 1% of all breast cancer cases (74). A successful treatment regimen for these cancers has not been established. Standard chemotherapy similar to basal-like or triple negative breast
cancer has been used since they express markers associated with basal breast cancer including EGFR and CK-5/6, but these cancers are usually chemoresistant (75). This led to further analysis of these cancers using a genomic-proteomic approach, which showed they were related to a group of cancers characterized by a claudin-low phenotype. These cancers had a loss of specific genes associated with cell-cell adhesion, as well as, markers associated with EMT such as TWIST1 and snail homolog 2 (SNAI2/SLUG). Furthermore, the PI3K/AKT pathway was highly activated compared to non-cancerous tissue (74). This activation is not common in basal-type cancers; therefore, targeting this pathway may be useful in treating these rare cancers (75).

Basal tumors are particularly aggressive, and women with basal-like breast cancer usually have poor relapse free survival (reviewed in (63)). Similar survival was observed in basal cancer as in HER2-positive breast cancer. Patients with triple negative breast cancer also have a poor outcome. High levels of metastasis to the bone (76) and the cerebellum (77) are common; furthermore, high levels of relapse are also widely found in triple negative breast cancer (78). In the USA, triple negative breast cancer seems to occur with higher incidence in premenopausal African-American women (79).

**Histological Classification of Breast Cancer**

Breast cancers can also be classified by their histology. These two types of classifications include ductal carcinoma *in situ*, which originates in the milk ducts, and lobular carcinoma *in situ*, which originates in the lobules (the milk producing glands) of the breast.
Ductal Carcinoma *In Situ*

Ductal carcinoma *in situ* (DCIS) is a non-invasive breast cancer where mammary ductal epithelial cells become malignant. DCIS can be readily identified by mammography or as a palpable mass. In a study done between 1988 and 1996 DCIS was present in 23% of the 200 cases studied (80). On the cellular level, DCIS involves malignant ductal epithelial cells that are proliferating within the duct, but do not cross the basement membrane (80). The common treatments for DCIS include mastectomy or lumpectomy (excision of the mass) with or without radiation therapy. DCIS can be either ERα-positive or ERα-negative; however, most are ERα-positive. Tamoxifen has also been shown to be helpful in about 50% of the cases, which is strong rationale for using this agent in women with ERα-positive DCIS (6, 80).

Lobular Carcinoma *In Situ*

Lobular carcinoma *in situ* (LCIS) is found in approximately 5% of all breast malignancies. Pathologically, this cancer can be identified by proliferation of malignant cells within the terminal duct lobular apparatus (reviewed in (81)). This structure is found at the ends of terminal ducts, which differentiate into lobules. The role of these ducts is to provide milk to the nipple (2). LCIS tumor cells are highly ERα-positive with low levels of mutations. About 90% of LCIS cases occur in premenopausal women. While LCIS is relatively benign, it has the potential to become invasive. Treatments for patients can range from non-operative endocrine therapies (such as tamoxifen) to bilateral mastectomy in the case of invasive tumors (81).
Invasive Lobular and Ductal Carcinoma

If left untreated both ductal and lobular carcinoma in situ can become invasive. Invasion is characterized by cancer cells moving beyond the basement membrane into the surrounding tissue. Pathologically these two carcinomas are distinct and will be described briefly below.

Invasive ductal carcinoma occurs in about 47%-79% of women worldwide (82) and between 55%-72% of patients with invasive ductal carcinoma are ERα-positive. These cancers also have higher amplification of HER2 and p21 than invasive lobular carcinoma (83, 84). Cytologically, the most concerning type of infiltrating ductal carcinoma consists of a “comedo” subtype, which is characterized by a large number of necrotic cells that fill the mammary duct as well as microcalcifications in this region.

Invasive lobular carcinoma occurs in about 3%-25% of women worldwide (82). Most (70%-92%) of these cancers are ERα-positive. This phenotype has been increasing rapidly especially in postmenopausal women and is difficult to diagnose (85). One reason is that invasive carcinoma is rarely characterized by a palpable mass. Morphologically, cells will infiltrate the stroma and surround the breast tissue (86). A study using microarray analysis comparing invasive ductal carcinoma and invasive lobular carcinoma showed that genes associated with cell adhesion/motility, fatty acid transport, and metabolism, as well as, genes associated with immune/defense response and electron transport were differentially regulated in the two types of cancer (85). Interestingly, invasive lobular carcinoma had a similar gene expression profile to normal like breast cancer (4-6). Since both types of cancer are positive for ERα, they may be treated with endocrine therapies (87).
**Conventional Classification of Breast Cancer**

**Inflammatory Breast Cancer**

Inflammatory breast cancer is characterized by rapid progression and a poor outcome (88). The name "inflammatory" breast cancer is misleading. The term comes from the reddened appearance of the breast, similar to what would be seen in an inflammatory response to infection, but here the reddened skin is actually caused by plugging of the lymphatics of the upper dermis with cancer cells. This type of breast cancer is rare and accounts for only 1-3% of breast cancers (89). It is a very aggressive disease, and surgery and radiation have little effect. About 62% of patients with inflammatory breast cancer have ERα-negative phenotype (90). Metastases occur in about 10-36% of cases at the time of diagnosis (88, 91, 92). However, if left untreated 90% of patients with inflammatory breast cancer may die within one year after diagnosis (93). Different combination chemotherapies have been used to treat these patients. Lopez summarizes various treatments including mastectomy, radiotherapy, hormonal therapy, Vincristine, Methotrexate and Vinblastine. The 5-year overall survival for these different treatments is given as 46% using a combination therapeutic approach (88).
Cancer Stem Cells

The History of Cancer Stem Cells

The idea of cancer stem cells (CSCs) dates back to the 1950s when studies identified stem cells in germinal cancer (teratocarcinomas). The first studies done by
Greene showed that embryonic tissue or cancerous tissue would grow in immune privileged sites of mice while normal tissue would not (106). Dilution studies showed that the tumor initiating cells were found at a frequency of 1:30-1:1000. These results were similar to the number of cells that survived chemotherapy. Later Salman et al. found that in adenocarcinoma the ratio of tumor initiating cells to normal tumor cells was between 1:1000- 1:100,000 (107). These cells could also form colonies in soft agar. Therefore, a foundation was set for many studies to come in the future on this topic (108).

Theories of Cancer Formation

Before discussing the theories of cancer formation, it is important to report the types of mutations that would need to occur in a normal cell in order to become a cancer cell. These are often called the hallmarks of cancer and include mechanisms for the cell to evade apoptosis, to be self-sufficient in growth signals, to have limitless replicative potential, to be able to sustain angiogenesis and to be able to metastasize (109). These qualities need to be achieved in order for a cell to become cancerous. Usually, this occurs by deregulation of signaling pathways associated with normal cell function (109).

Stochastic theory

In the traditional model of carcinogenesis (often called the stochastic model), cancer occurs through a series of mutations caused by environmental or other factors that lead to genetic instability (110). This model states that mutations can occur in any cell, which will become transformed given that the appropriate mutations occur (reviewed in (111)). This model also states that a tumor is relatively homogeneous and that the pathways that lead to tumorigenesis are active in all tumor cells. Therefore, it would be difficult to find the exact cell from which a particular tumor originated because
virtually any cell within the appropriate lineage would have an equal probability of initiating that tumor.

Another model that is related to the stochastic model is the clonal evolution model. In this model, certain cells are given a survival advantage (because of mutations). These cells will be selected for and survive while normal cells will die. The mutated cells produce daughter cells leading to tumor growth. Tumor heterogeneity is due to aberrant differentiation, impact from epigenetic variation, and alterations due to the microenvironment.

However, the stochastic model cannot explain some other aspects of tumors. First, numerous mutations are required to fully transform a cell, and it would take longer than the normal life span of a typical cell to acquire these mutations. Second, if tumors are derived from a single transformed cell, cancers would be fairly homogenous. Yet, it is well recognized that breast tumors are highly heterogeneous. Third, cancers can recur despite treatment that appears to completely eliminate the tumor. This suggests that highly tumorigenic cancer cells are able to evade death caused by chemotherapy and radiation. These cells can later proliferate to cause recurrent or metastatic disease. As discussed below, the cancer stem cell theory appears to address these issues.

The cancer stem cell theory

Another model for tumor development is the cancer stem cell (CSC) model (also known as the hierarchical model). This model states that a subset of tumor cells, termed CSCs, may be ultimately responsible for cancer initiation and recurrence (reviewed in (112)). These cells can self-renew and differentiate, which is thought to be responsible
for tumor heterogeneity. Self-renewal is defined as the ability to produce an exact copy of a cell, which has the same ability to proliferate, differentiate and expand as the parental cell (113). Progenitor cells or fully differentiated cells in the tumor do not have this capability. The CSCs possess several other characteristics of normal tissue stem cells including indefinite proliferation, slow replication, and production of daughter cells that undergo multilineage differentiation. The daughter cells make up the bulk of the tumor and are non-tumorigenic, while the CSCs are tumorigenic and make up a small proportion of the tumor, though this is controversial and will be discussed in detail in a separate section. CSCs are also capable of unlimited proliferation. It is hypothesized that CSCs are able to evade killing by traditional therapeutic approaches by utilizing some of the same properties that protect normal stem cells from these agents. For example, current therapies often target rapidly proliferating cells; however, CSCs, like normal stem cells, are believed to divide slowly and can even enter quiescence. Furthermore, CSCs and normal stem cells express higher levels of anti-apoptotic proteins than non-stem cells and express higher levels of multidrug resistant transporters called ATP Binding Cassette (ABC) transporters than non-stem cells, which effectively efflux toxins from the cell (114). Together, these characteristics may protect the CSCs from traditional therapies allowing them to remain unharmed after destruction of the bulk tumor cells and capable of causing relapse or metastases.

It is clear that CSC possess qualities that may make them more suitable for transformation. First, they are long-lived, which gives them time to acquire mutations, and secondly, they may need to acquire fewer mutations in order to become cancerous. For example, stem cells have the ability to migrate (a quality necessary for metastasis), and they are resistant to apoptotic signals and have limitless proliferation potential.
These cells have already fulfilled some of the hallmarks of cancer without acquiring any mutations. Therefore, they would need a smaller number of mutations in order for transformation to occur and since they are long-lived; they have the time to acquire them.

The CSC model may also explain why tumors are heterogeneous. Dontu et al. proposed a theory where CSCs can develop from ERα-negative stem cells or ERα-positive progenitor cells. An ERα-negative stem cell can become mutated, and lead to the formation of an ERα-negative CSC (33). This CSC may self-renew producing additional ERα-negative CSCs or differentiate into ERα-negative cancer cells. These types of cancers are poorly differentiated and usually found in the basal group when characterized by molecular profiling. Mutations can also occur in the progenitor cells leading to the formation of either ERα-positive or negative CSCs. In this case tumors would contain both ERα-positive and ERα-negative cancer cells. This theory can help explain problems with treatments. These tumors are likely to recur if treated with anti-estrogens, for example, because the ERα-negative stem cell would not be affected by these treatments and would produce other ERα-positive or negative cancer cells. ERα-positive cells can also arise from ERα-positive progenitor cells that have re-acquired self-renewal and mutated into CSCs. ERα-positive tumors could therefore be derived from either an ERα-negative CSC where the daughter cells differentiate down an ERα-positive pathway or from an ERα-positive CSC. These types of tumors are well-differentiated and may respond well to anti-estrogen therapies (33).
The Origin of CSCs.

CSCs may arise from stem cells, progenitor cells or somatic cells that accumulate genetic mutations that result in deregulated self-renewal and production of daughter cells that undergo aberrant differentiation (115). Several lines of evidence support each of these possibilities. Normal tissue stem cells are already capable of indefinite self-renewal and can give rise to more differentiated progenitors making them an obvious possible source of CSCs. They are also long-lived and would be capable of accumulating the serial mutations required for transformation over the lifetime of the cell. Finally, CSCs tend to express markers associated with normal tissue stem cells such as CD133, nestin, c-kit, sox2, oct4 and musashi-1 (116-119).

Alternatively, CSCs may be derived from progenitor cells. Although relatively immature, progenitor cells have lost the ability to self-renew and would need to re-acquire this ability to generate a CSC. Recent evidence indicates this may be possible, at least in leukemic cells, where the introduction of oncogenic fusion gene products into hematopoietic progenitor cells resulted in acute myeloid leukemia in animal models. Cozzio et al. found that expression of the MLL-ENL fusion gene product in hematopoietic progenitor cells resulted in leukemia, albeit with less efficiency than when expressed in true hematopoietic stem cells (120). Similar results were also found with the MOZ-TIF2 fusion gene product (121). However, these studies were performed in a mouse model. Murine cells are known to be more readily transformed than human cells. Therefore, it is unclear if similar results are possible using progenitor cells from the more complex human system.
Finally, somatic cells may undergo mutations resulting in re-programming to a multipotent stem cell phenotype. Recent studies indicate that cells called induced pluripotent stem cells could be obtained from normal fibroblasts by transduction with specific factors (Oct3/4, sox2, Klf4, and c-Myc). When these factors were added to differentiated fibroblasts the resulting cells were very similar to human embryonic stem cell in morphology, cell surface markers, proliferation and telomerase activity (122). Similarly, Yu et al. showed that expression of oct4, sox2, nanog, and LIN28 in human dermal fibroblasts converts them into pluripotent cells with a phenotype virtually indistinguishable from embryonic stem cells (123). Although experimental evidence indicates that CSCs may be derived from any of these cells (stem, progenitor or fully differentiated somatic cells), it is currently unknown, which is the origin of breast CSCs. Indeed, it is possible that breast CSCs are derived from all three types of cells depending upon the genetic and epigenetic characteristics of an individual tumor. These possibilities are summarized in figure 3.
The Stem Cell Niche

As mentioned above stem cells have the ability to self-renew, differentiate, or remain quiescent. The signals that drive these decisions come from the surrounding microenvironment referred to as the stem cell niche. The niche is made up of a specialized group of cells that interact with the stem cell and are responsible for its maintenance. The niche is found in a specific location in normal tissues. For example, the skin stem cell niche is located in the hair follicle (124). It is unclear where the breast stem cell niche resides. Stem cells are physically attached to the niche by various adhesion proteins. Extrinsic signals will trigger the niche to cause the stem cells to differentiate. Until these signals are received, the stem cell will be maintained in a
quiescent state. A niche for the CSC has also been identified for several cancers. The
niche is thought to maintain self-renewal and differentiation of the CSC, as well as,
protect these cells from apoptotic agents (125). Neural stem cells occupy regions called
"vascular niches" which are rich in blood vessels and lined by endothelial cells.
Endothelial cells are thought to secrete factors that promote self-renewal and survival of
CSCs (125). Interestingly, disruption of the niche may be one way of targeting CSCs,
since this would cause differentiation of the CSCs. Calabrese et al. showed that after
transplanting medulloblastoma cells in the presence or absence of endothelial cells
resulted in tumors with up to 25 times higher levels of CSCs in the presence of
endothelial cells than when they were absent (126).

Identification and Isolation of Normal Stem Cells and CSCs in vitro

The identification and isolation / enrichment of normal stem cells was a major
advancement in the ability to test the cancer stem cell theory. Currently, four different
methodologies have been successfully used: side population (SP) (127), sphere
formation (128), cell surface markers (129, 130), and aldehyde dehydrogenase (ALDH)
activity (131). It is important to note that these methodologies can identify / isolate either
normal stem cells or CSCs from various tissues.

Side population

As stated earlier, stem cells and CSCs express high levels of multidrug resistant
transporters (ABC transporters). These transporters can efflux different substrates
including lipids, metabolites, and ions as well as a variety of drugs including
chemotherapeutic drugs making cells resistant to treatment (132-134). Researchers
have taken advantage of the fact that these transporters also efflux vital fluorescent
dyes, including Hoescht 33342 and DyeCycle Violet (135). Bulk cell populations can be loaded with dye, which is effluxed from the stem cells within the sample via the ABC transporters (136, 137). These cells become less fluorescent than the bulk cell population and can be readily identified on flow cytometry and collected through fluorescence-activated cell sorting (FACS). As the stem cells (or CSCs) are the small, less fluorescent population at the side of the majority of cells, they are termed the SP. To confirm identification of the SP, drugs that block ABC transporter activity, such as verapamil hydrochloride or reserpine, can be used to prevent efflux of the dye (127). Under these conditions, the stem cells / CSCs retain the dye and the SP will “disappear” as the cells remain fluorescent (since their efflux channels are blocked) and sort with the bulk cell population.

Evidence has confirmed that SP cells are enriched for stem cells. Alvi et al. showed that the SP isolated from normal mammary tissue were undifferentiated, but could be prompted to differentiate into epithelial or myoepithelial cells in culture (136). Reverse transcriptase polymerase chain reaction (RT-PCR) analysis has confirmed increased expression of several ABC transporters in SP cells derived from normal breast tissue compared to non-SP (136). In addition, SP cells derived from the MCF7 breast cancer cell line were shown to be more tumorigenic than the non-SP (137). When 1,000 SP cells collected from the MCF7 cell line were injected into mammary fat pads of mice, tumors reproducibly formed, while injection of tens of thousands of non-SP cells could not produce a tumor (137).

Further evidence has specifically linked the ABCG2 (BCRP1) channel in conferring the SP phenotype in both hematopoietic progenitors derived from lung, as
well as, human embryonic kidney cells (HEK293) and bone marrow (30). In one study, SP cells were isolated from murine bone marrow using FACS. Elevated expression of ABCG2 in the SP compared to non-SP cells was confirmed by RT-PCR along with lower expression levels of other transporter proteins ABCC1 (MRP1), ABCC2 (MRP2), ABCC3 (MRP3) and ABCC4 (MRP4) (30). Scharenberg et al. showed that the expression of the ABCG2 transporter was sufficient to confer the SP in hematopoietic stem cells (138).

**Mammosphere formation**

Maintenance of stem cells in an undifferentiated state in culture has only recently been possible due to the development of specialized non-adherent culture conditions. In work pioneered by Reynolds and Weiss, neural stem cells were grown as neurospheres where about 20% of sphere cells were able to self-renew (form spheres) and differentiate along multiple lineages (139, 140). Dontu and colleagues modified this system to successfully culture mammary stem cells and breast CSCs as mammospheres (128). Here, bulk cell populations from reduction mammoplasties (normal breast tissue) were plated on ultra-low adhesion tissue culture plates in serum free media supplemented with growth factors (EGF, bFGF), protein supplement B27, and heparin (128). To confirm the spheres were composed of stem and progenitor cells, mammospheres were immunostained for expression of markers associated with luminal (epithelial specific antigen (ESA), Muc-1, CK-18), myoepithelial (CD10, α-smooth muscle actin, α-integrin 6), and progenitor cells (CK-5, CK-14) (141). They observed that Muc-1, α-smooth muscle actin, and CK-18 were not found in mammospheres. CD10, CK-5 and α-integrin 6 were present in a few cells and ESA (50% of cells) and CK-14 (30% of cells) were randomly dispersed (128). They determined that each sphere was derived from a single mammary stem cell having the ability to self-renew and differentiate along any of
three mammary-related lineages: alveolar epithelial, luminal epithelial or myoepithelial.

Each mammospheres was composed of 1-2 multipotent stem cells surrounded by bipotent and tripotent progenitor cells (128). Mammospheres were also shown to be more tumorigenic than bulk cultured cells. Grimshaw et al. showed that injecting only 5,000 mammosphere cells derived from a breast cancer pleural effusion was sufficient to produce tumors in non-obese diabetic severe combined immune deficient (NOD/SCID) mice (142). Tumor formation was correlated with samples that could form large mammospheres (142).

**CD44**\(^{+}\) **CD24**\(^{-}/lo\)w **ESA**\(^{+}\) and **CD133 markers**

Another way of identifying breast CSCs is to examine expression of cell surface markers including CD44, CD24, and ESA. Cells derived from either xenograft tumors or breast cancer pleural effusion samples that have the **CD44**\(^{+}\) **CD24**\(^{-}/lo\)w **Lineage**\(^{-}\) **ESA**\(^{+}\) phenotype were shown to be more tumorigenic than cells expressing other combinations of CD44, CD24, and ESA (129). As few as 200 cells with the **CD44**\(^{+}\) **CD24**\(^{-}/lo\)w **Lineage**\(^{-}\) **ESA**\(^{+}\) phenotype isolated from primary breast cancers were required to reproducibly initiate tumor formation in mice (129). Furthermore, once tumors were established, the **CD44**\(^{+}\) **CD24**\(^{-}/lo\)w **Lineage**\(^{-}\) **ESA**\(^{+}\) cells could be re-isolated from the tumor and serially passaged into naïve animals indicating this population was capable of self-renewal (143).

In a study by Sheridan et al. the **CD44**\(^{+}\) **CD24**\(^{-}/lo\)w was isolated from various breast cancer cell lines (MCF7, T47D, MDA-MB-231, MDA-MB-468, and SKBR3 among others). This phenotype was found in mainly basal/mesenchymal or myoepithelial cells but not luminal cells (144). These cells also contained certain genes associated with
metastasis such as interleukin-6, interleukin-8 and urokinase plasminogen activator. Different cell lines tested showed that the $CD44^+ CD24^{-/low}$ phenotype was consistent with invasion. However, this phenotype was not responsible for homing and proliferation at the site of metastasis (144). Fillmore and Kuperwasser showed that the $CD44^+ CD24^{-/low}$ ESA$^+$ phenotype derived from breast cancer cell lines correlated well with tumorigenic potential while the $CD44^+ CD24^{-low}$ phenotype did not. Breast cancer cell lines derived from luminal cells (such as MCF7 and SUM225) contained very low levels ($<1\%$) of this phenotype while breast cancer cell lines derived from basal cells (MDA-MB-231, SUM1315 and SUM 159) contained higher levels ($>1\%$). These cells were also shown to be more tumorigenic than $CD44^+ CD24^{-low}$ ESA$^-\$ cells (145). In another study, no correlation was observed between tumorigenicity and the percentage of $CD44^+ CD24^{low/-}$ cells in pleural effusions from metastatic breast cancer patients (142).

In a clinical study, breast cancer patients on chemotherapy (Docetaxel, or Doxorubicin and Cyclophosphamide) were monitored for the presence of the $CD44^+ CD24^{-low}$ phenotype in biopsy samples taken before, during and after their treatment. The results showed that tumors had much higher levels of $CD44^+ CD24^{-low}$ after chemotherapy than before treatment (146). This indicated the cells were resistant to chemotherapeutic agents, which killed off the majority of the bulk tumor cells leaving the putative CSC intact. The authors also showed an increase in mammosphere efficiency when the $CD44^+ CD24^{-low}$ fraction was grown as mammospheres (146).

The $CD44^+ CD24^{-low}$ population was also shown to be more resistant to radiation therapy. Phillips et al. found that MCF7 and MDA-MB-231 cells grown as mammospheres (which contained elevated levels of $CD44^+ CD24^{-low}$ compared to cells
grown in a monolayer) had elevated levels of Notch-1 after irradiation (147). Mammospheres were also much more resistant to radiation than cells grown in a monolayer. Additionally, the CD44⁺ CD24⁻/low phenotype increased significantly in the mammosphere population after treatment with radiation, however, that was not the case for the adherent population (147).

Finally, using a genomics-proteomics approach the gene expression patterns of CD44⁺CD24⁻/low and the different molecular classifications of breast cancer were identified. It was found that metaplastic breast cancer (claudin-low) had a high ratio of the CD44⁺/CD24⁻/low- phenotype. In addition, the CD44⁺ CD24⁻/low genomic profile correlated with the metaplastic breast cancer and claudin-low profiles (75). This indicates that these cancers express high levels of genes associated with the stem-like phenotype.

More recently, CD133, a known marker of hematopoietic stem cells has been shown to be co-expressed with high levels of stem cell-regulatory genes in breast cancer stem-like cells (130, 148). It should be noted that the CD133 marker does not only identify breast CSCs but also glioma, brain, and prostate CSCs (149, 150). Wright et al. used cell lines derived from human tumor xenografts to test the presence of CD44⁺ CD24⁻/low and CD133-positive. They found that in cell lines derived from a tumor that had low levels of CD44⁺ CD24⁻/low contained higher levels of CD133-positive cells (between 2 to 6%) (130). CD133-positive cells were also more tumorigenic than CD133-negative cells requiring only 50-100 cells to initiate a tumor in NOD/SCID mice. Although fewer reports have utilized CD133 as a marker for breast CSCs, it appears this marker may be useful, particularly in cases where CD44, CD24 staining has not been helpful.
Aldehyde dehydrogenase-1 (ALDH) activity

ALDH, an enzyme that oxidizes intracellular aldehydes, has been described as a putative marker for normal stem cells and CSCs (131, 151-153). Based on sequence analysis, three classes of mammalian ALDHs have been identified: ALDH-1, -2, and -3. Classes 1 and 3 contain ALDH isoforms that are both constitutively expressed and inducible. Class 2 isoforms are constitutively expressed in the mitochondria. Each class of ALDH appears to oxidize a variety of different substrates. Of importance to our work, the Class 1 isoform A1 (ALDH1A1) has been identified as highly expressed in embryonal tissue as well as in adult stem cells (154).

ALDH appears to be responsible for oxidizing retinol to retinoic acid, which is important in early stem cell differentiation (155), and it may also be involved in chemoresistance (156). High levels of ALDH-1 were found in metastatic breast cancers and shown to cause resistance to the chemotherapeutic agent cyclophosphamide (157). High ALDH-1 activity has been associated with murine hematopoietic stem cells from bone marrow or neural stem cells (158, 159). Cells containing elevated levels of ALDH-1 have also been identified in leukemia, multiple myeloma, and breast tumors (153).

Ginestier et al. identified and isolated breast CSCs using the Aldefluor assay where the ALDH-1 enzyme converts the substrate BODIPY-aminoacetaldehyde into the fluorescent product BODIPY-aminoacetate which can then be analyzed by flow cytometry (131). Stem-like cells become highly fluorescent due to increased ALDH-1 activity and are readily detectable by flow cytometry. Ginestier et al. showed that only the Aldefluor-positive, but not the Aldefluor-negative cell population, could form mammospheres in culture and tumors in mice, even when injected at low cell numbers. In fact, using
Aldefluor-positive cells that were also CD44+ CD24low/− Lineage−, they found that as few as 20 cells were able to generate a tumor. In contrast, Aldefluor-negative cells that were CD44+ CD24− Lineage− were unable to form tumors when 50,000 cells were implanted (131).

The Cancer Stem Cell hypothesis and multidrug resistance

The CSC hypothesis may explain why resistance occurs in breast cancer. Two types of resistance exist: inherent and acquired. Stem-cells have inherent resistance as they possess channels that will allow them to efflux various toxins. As stem-cells divide they produce progenitor cells which are sensitive to various drugs. However, in aggressive types of cancer these progenitor cells or differentiated may acquire resistance via gene amplification or rearrangement, to various drugs and be given a survival advantage leading to acquired resistance. In this case, certain therapies may become ineffective after prolonged treatment (160).

The Cancer Stem Cell Hypothesis Controversy

The CSC hypothesis remains controversial. First, some investigators argue that the hypothesis itself is incorrect and maintain that cancer follows the stochastic theory. This argument is based on the idea that every cancer cell is equally tumorigenic. When human tumor cells are injected into mice, even in orthotopic models, the cells are required to grow in a foreign and potentially adverse environment. Some tumor cells may simple be unable to grow under these conditions. For example, the CD44+CD24low− subset of human breast cancer cells may simple be better able to survive in the murine mammary fat pad than their CD44+CD24+ counterparts. This viewpoint was supported by studies by Kelly et al. who used primary murine lymphoma cells derived from E-myc
transgenic mice and implanted them into the same strain of mouse (161). They found that as few as 10 lymphoma cells could form tumors under these conditions. Moreover, they found that lymphoma cells with a murine stem cell phenotype (Sca$^+$ AA4.1$^{High}$) or a non-stem phenotype (Sca$^+$ AA4.1$^{low}$) were equally as effective at initiating lymphoma in mice. Supporters of the CSC theory, however, argue that the Eu-myc transgenic model system used in these experiments may have influenced the results. These mice overexpressed the oncogene c-Myc, and the model was chosen as a means to accelerate the process of tumorigenesis. However, c-Myc has been identified as one of the key genes involved in reverting fully differentiated fibroblast into cells with a stem phenotype. Thus, overexpression of Myc in the tumor cells may have played a role in dedifferentiation of the cells toward a stem-like phenotype giving them a growth advantage.

Another CSC controversy is whether or not the CSC must be a rare subset of the tumor cells. The original definitions of CSCs stated that they were less than 1% of the total tumor cell population. Yet, several studies have found cells with the stem phenotype are actually quite common in some tumors or cancer cell lines. For example, Al Hajj et al showed that between 11 and 35% of breast cancer cells in pleural effusions possessed the CD44$^+$ CD24$^{low/-}$ phenotype. However, breast cancer pleural effusions represent aggressive, metastatic disease, and it has been proposed that more advanced, aggressive cancers likely contain a higher proportion of stem-like cells.

Although not strictly related to the CSC hypothesis, the utility of SP in stem cell studies is also controversial. As stated earlier, the SP is identified as the subset of cells that do not maintain a vital fluorescent dye, such as Hoechst 33324. As stem cells can
actively efflux these dyes via ABC transporters, it was hypothesized that they could be enriched by collecting the non-fluorescent subset of Hoechst-stained cells on flow cytometry. Indeed, SP cells from mammary tissue are enriched for stem cell markers, lack differentiation markers and are more tumorigenic in animal models than non-SP cells (136, 137, 162). While some investigators have reported that the normal mammary stem cells that can generate mammary glands in mice are not found in the SP (32) other investigators have found that the SP proliferates rapidly when the ducts are being formed, but remains quiescent in the adult mouse (29). Furthermore, when the SP is transplanted the progeny can form ductal and alveolar structures (29). This suggests that the SP contains the stem/progenitor cell population. In addition, concerns have been raised regarding the toxicity of the dyes in cells that do not exclude it, as this may alter interpretation of functional assays performed with SP and non-SP cells. As SP analysis was one of the first methods for identifying stem-like cells, it is still widely utilized. However, the development of other techniques that do not have these problems have led many investigators away from this technique.

As self-renewal and the capacity to differentiate along multiple lineages are the hallmarks of stem cells, assays capable of measuring these factors are the best assays for CSC studies. The gold standard for demonstrating CSCs is serial transplantation in an animal model. Here, the CSC subset is injected into the animal, a tumor is formed (which can be evaluated for heterogeneity), and the CSCs re-isolated and injected into a naive animal (showing self-renewal). As these assays can take long periods of time, in vitro assays such as sphere formation and colony formation assays have become mainstays in the field.
The Notch Signaling Pathway

As mentioned above one way of eliminating CSCs would be to target pathways that are dysregulated in CSCs. The Notch pathway is one such pathway that is dysregulated in both CSCs and breast cancer.

The Structure of Notch

The Notch receptor was first described in *Drosophila* by Thomas Hunt Morgan. He noticed a unique notched phenotype in the wings of *Drosophila* (163). Later it was discovered that this phenotype was due to a haploinsufficiency of the Notch gene. The Notch gene in *Drosophila* was cloned by Artavanis-Tsakonas and Young in 1985 (164, 165). In mammals there are four Notch receptors (Notch-1,-2,-3,-4) and five Notch ligands (Delta-like (Dll) -1, -3, -4, and Jagged (Jag)-1,-2) (reviewed in (166)). The ligands are homologous to the *Drosophila* ligands Delta and Serrate.

The Notch receptor is a heterodimer and consists of an extracellular portion and a transmembrane region (163). The extracellular portion is composed of epidermal growth factor (EGF)-like repeats, which are involved in ligand binding and three cysteine-rich Notch/LIN12 repeats (LN) that prevent Notch activation in the absence of ligand binding. The intracellular portion of Notch contains a RAM domain, six ankyrin repeats (ANK), two nuclear localization signals (NLS), a transcription transactivation domain (TAD) and a PEST (proline, glutamate, serine, and threonine) sequence, which is important in mediating Notch degradation. The different Notch receptors have a very similar structure but vary in the number of EGF-repeats. Notch-1 and Notch-2 contain 36 EGF repeats while Notch-3 has 34 EGF repeats and Notch-4 only 29 EGF repeats. Additionally, Notch-1 contains a strong TAD region while Notch-2 contains a weak TAD,
and Notch-3 and Notch-4 do not have any TAD regions. The Dll and Jag ligands also contain EGF-like repeats and a DSL region (Delta, Serrate and LAG-2), which will interact with Notch at the amino-terminal end. Figure 4 summarizes the structure of the different Notch receptors and ligands.

**Figure 4:** A summary of the structure of the four Notch receptors. Each receptor consists of an intracellular and extracellular portion. The extracellular region contains EGF repeats that interact with the EGF-like repeats of the Dll and Jag ligands. EGF = epidermal growth factor, LN = cysteine-rich Notch/LIN12 repeats, ANK = ankyrin repeats, TAD = transactivation domain, NLS = nuclear localization signal, PEST = proline, glutamate, serine and threonine, DSL = Delta, Serrate, Lag1, Dll = Delta-like, Jag = Jagged, CR = cysteine rich region. Modified from Radtke and Raj (163).

**Notch signaling**

The Notch signaling pathway is an evolutionarily conserved signal transduction mechanism by which neighboring cells can communicate with one another. The precursor to the Notch receptor is formed in the endoplasmic reticulum then is transported to the Golgi apparatus where it is cleaved by a furin protease (167). This
cleavage produces the extracellular domain and the transmembrane domain. The two are associated in a non-covalent calcium dependant interaction. The extracellular domain binds the ligand and the intracellular domain is required for further signaling after ligand binding. The Notch protein is transported to the membrane. Ligand-receptor interaction triggers two successive proteolytic cleavages of the receptor; first by ADAM 10/17 (a disintegrin and metalloprotease) and then by the γ-secretase complex. After the first cleavage the extracellular portion is endocytosed into the ligand bearing cell (168). The second cleavage releases the intracellular portion of the receptor (N\text{IC}), which is called the active form of Notch. N\text{IC} translocates to the nucleus where it binds to the CSL (CBF-1/RBP-Jk (mammalian), Suppressor of Hairless (Drosophila), Lag-1, (Caenorhabditis elegans)) transcription factor, via its RAM23 domain and displaces the co-repressor complex (including silencing mediator of retinoid and thyroid hormone receptors (SMRT), histone deacetylase (HDAC-1), and Ski-interacting protein, (SKIP), CBF-1 interacting corepressor (CIR), and SMRT/HDAC-1 associated repressor protein (SHARP)) (169). RAM and TAD are involved in the binding of the activated form of Notch to CBF-1. Ankyrin repeats further stabilize the interaction between N\text{IC}, CBF-1 and the DNA. A co-activator complex consisting of mastermind-like 1 (MAML1), histone acetyltransferases (p300, PCAF/GCN5 for example) is then recruited leading to transcriptional activation and expression of various target genes including two families of transcription factors, hairy enhancer of split (Hes), and hairy/enhancer of split related with YRPW motif (Hey) (170-173). The Hes and Hey family are a group of basic helix-loop-helix transcription factors that are involved in repressing expression of various activators of cellular differentiation. Studies have demonstrated that at least Hes-1, Hey-1, and Hey-2 are primary targets of Notch activation (170-173). The Notch pathway is summarized in Figure 5. There are many other Notch targets including p21\text{Cip/Waf}, cyclin
D1, cyclin A, and NF-κB (reviewed in (174)). Some negative regulators of Notch include Numb, which causes endocytosis of Notch followed by its degradation in the proteosome. Notch can also be degraded by one of several E3 ubiquitin ligases including Deltex (175). It is important to note that Notch activation of downstream target genes is context dependant, that is, different Notch targets can be upregulated depending on the cell type, the Notch receptor and ligand involved in signaling and the density of the receptors and ligands.

Notch can also signal via a non-canonical signaling pathway, which is independent of CBF-1. Some targets of Notch signaling that are modulated independently of CBF-1 include β-catenin, hypoxia inducible factor-1α and Deltex. Interestingly, Deltex is a downstream target of Notch, which then binds to Notch along with β-arrestin and leads to its ubiquitination and degradation.
Notch acts as an oncogene in breast cancer. It may play a role in tumorigenesis by increasing proliferation or preventing differentiation of cells (reviewed in (174)).

Several oncogenic targets of Notch include cyclins A, D1, and D3, HER2, and NF-κB (reviewed in (174)). Several studies have been done in order to elucidate the roles of Notch in cancer. The first studies examined mice with mammary tumors caused by the murine mammary tumor virus (MMTV). In 20% of tumors, the MMTV genome integrated into the Notch-4 gene causing overexpression of a truncated Notch-4. The enhancer sequences in the viral long terminal repeats promoted transcription of the intracellular
portion of Notch 4, which is functionally active. Thus, the cells constitutively expressed
activated Notch-4 resulting in altered proliferation of immature ductal cells. Similarly, the
Notch-1 gene was identified as a target of MMTV insertion (reviewed in (176)).

In human breast cancers, Notch expression is also elevated. In one study, seven
primary breast ductal carcinomas were immunostained for Notch-1 and showed high
levels of Notch-1 expression compared to normal breast cells (177). In these samples,
four of the seven samples stained positive for the h-Ras oncogene. Those that stained
positive for h-Ras showed strong but diffuse staining for Notch-1, whereas, h-RasV12
negative cells showed only a few cells that had strong staining for Notch-1 (177).

Reedijck et al. used in situ hybridization studies to show that elevated Notch-1
and Jag-1 were associated with poor prognosis in breast tumors from patients (178).
Patients that had tumors with high Jag-1 expression had a 5-year survival rate of 42%
versus 65% for patients with low Jag-1 expression with a median survival of 50 months
for the former and 83 months for the latter. Some tumors (12/40, 30%) with high levels of
Jag-1 belonged to the basal subtype of breast cancer while the majority did not (28/40,
70%). Elevated Jag-1 was a predictor of poor outcome regardless of the type of breast
cancer (178). This laboratory confirmed their work using an additional 887 breast cancer
samples. They found elevated Jag-1 expression was associated with basal-type breast
cancer and that disease free survival was significantly reduced when Jag-1 was
overexpressed at both the mRNA and protein levels (179).

Cohen et al. further looked at the effect of Jag mediated Notch signaling in breast
cancer (180). They identified that triple-negative breast cancer expressed higher levels
of Jag-1 compared to other types of breast cancer cell lines. Knockdown of Jag-1 using an siRNA approach resulted in a significant reduction in proliferation. Microarray profiling of triple-negative breast cancer cell lines (HCC1143, MDA-MB-231) transfected with siJag-1 was done and a comparison made against control transfected cells. The authors determined that cyclin D1 was highly downregulated in siJag-1 transfected MDA-MB-231 cells. Additionally, when Notch-1 and/or Notch-3 were knocked-down, cyclin D1 expression was also reduced. Notch-3 had a much greater effect on levels of cyclin D1 than Notch-1 (180).

Stylianou et al. studied the expression of Notch receptors and ligands in various breast tumors and breast cancer cell lines and found further evidence for aberrant Notch signaling in breast cancer (181). They showed Notch-1, Notch-3, Dll-4, Jag-1 and Jag-2 were expressed in normal breast epithelial cells. Expression of Notch-2, Notch-4, Dll-1 and Dll-3 were not examined. Using immunohistochemistry, they showed that Notch was expressed in the luminal epithelium. Next, they stably transfected immortalized normal breast epithelial cells (MCF10A) with a plasmid containing activated Notch-1. They showed these transformed cells grew uncontrollably in culture, formed colonies well in soft agar, and apoptosis was not induced in transformed cells treated with the kinase inhibitor staurosporine, or DNA damaging agents melphalan and mitoxantrone while there were high levels of apoptosis in untransformed cells as well as growth inhibition and poor colony formation. In addition, a panel of breast tumors and cell lines of various breast cancer phenotypes was studied for the expression of activated Notch. All samples tested showed increases in activated Notch and downstream target Hey-1 by Western blotting. Finally, the authors showed that by overexpressing, Numb, a negative regulator of Notch, they could reverse the transformation of the cell lines tested (181).
separate study, Pece et al. showed that Numb protein expression as determined by immunohistochemistry was shown to be decreased in 50% of 321 breast cancer patient samples tested (182).

Rizzo et al. showed that Notch-1 or Notch-4 levels were elevated in various infiltrating breast tumor samples compared to normal breast tissue using immunohistochemistry (183). ERα-negative cell lines (MDA-MB-231, and T47D-C42) showed higher levels of Notch activation than ERα-positive cell lines (MCF7, T47D-A18) or the HER2 overexpressing cell line SKBR3. The data also showed that estrogen inhibits Notch signaling (183). The authors proposed that estrogen inhibits Notch signaling, in part, through ERα by inhibiting γ-secretase cleavage of Notch. Moreover, Notch-1 was shown to induce Notch-4 expression in MCF7 cells. Notch-4 expression was associated with Ki67 (a proliferation marker) in primary tumor samples. In other words, cells expressing Notch-4 were also proliferating; therefore, Notch-4 may be a therapeutic target for breast cancer. In MCF7 cells, inhibition of ERα (by anti-estrogens, estrogen deprivation, or loss of ER) led to higher levels of Notch-1 activity when compared to conditions where estrogen was present; this induced expression of Notch-4 and lead to an increase in proliferation and invasion of these cells. The exact mechanism for invasion was not defined but in the absence of Notch-1 or Notch-4 led to decreased invasion of MDA-MB-231 cells and introduction of the activated form of Notch-1 (N\text{IC}1) in MCF7 cells increased invasion through matrigel.

Osipo et al. demonstrated that overexpression of HER2 suppressed Notch-1 activity and treatment with trastuzumab or a dual EGFR/HER2 tyrosine kinase inhibitor reversed this effect (184). Furthermore, they showed that cell lines, including BT474,
SKBR3 and MCF7/HER2-18 overexpressing HER2, had low levels of Notch activity as measured by a CBF-1 luciferase reporter assay compared to MDA-MB-231 cells and MCF7/Neo cells. In the presence of trastuzumab, Notch-1 activity was increased as were Notch targets Hey-1, Hes-1 and Deltex-1. In the presence of γ-secretase inhibitors and trastuzumab, Notch activity was decreased and apoptosis increased by 20%-30% in trastuzumab sensitive BT474 cells. These data suggest that Notch-1 might play a role in the resistance of breast cancer cells to trastuzumab, which can be reversed by inhibiting Notch-1 (184).

Finally, Notch-3 was shown to have a critical role in HER2-negative breast cancer cells. Yamaguchi et al showed that in breast cancer cell lines that were HER2-negative (MCF7, HCC1419, and MDA-MB-468) expressed higher levels of Notch-1 and Notch-3 than HER2-positive cells. When Notch-3 was specifically inhibited using siRNA a significant decrease in cell proliferation in HER2-negative cells was observed, but this effect was only seen in one HER2-positive cell line (HCC1419). However, a knockdown of Notch-1 did not have this effect. Furthermore, apoptosis was induced in a HER2-negative breast cancer cell line after transfection with siNotch-3. Similar results were observed when Jag-1, or Jag-2 were knocked-down. These data suggest that Notch-3 is important in the proliferation and survival of HER2-negative breast cancer cells and may be a therapeutic target to attempt to eliminate this type of cancer (185).

**Gamma-Secretase Inhibitors**

γ-secretase is a multi-subunit protease which consists of a catalytic subunit (presenilin-1, presenilin-2), Pen-2, Aph1 and Nicastrin (186). γ-secretase is required for the activation of all four Notch receptors, and γ-secretase inhibitors (GSIs) have been
shown as effective agents in blocking Notch signaling. At this point, it should be noted that γ-secretase not only cleaves Notch, but also has other intramembranous targets including CD44, E-cadherin and ERBB4 (186). Given the link between Notch and cancer, inhibition of Notch signaling has been proposed as a strategy for cancer treatment. Studies using GSIs have shown dramatic reductions in tumor burden in xenograft models (187, 188). Based on these promising results, GSIs have now moved into Phase I clinical trials in breast cancer.

Several studies have illustrated the effectiveness of using GSIs to treat cancers. Before going into details it is important to mention that these studies used at least one of the following GSIs: Z-Leu-Leu-Nle-CHO (LLNle, EMD Biosciences), LY411,575, or MRK003. LLNle is a tripeptide GSI, which has an aldehyde group that can inhibit serine proteases including calpain when used at high concentrations (187). This GSI has also been shown to inhibit the proteosome in breast cancers at concentrations higher than 10 µM (L. Miele unpublished data). Therefore, this drug must be used at lower concentrations to inhibit Notch signaling without blocking proteosome activity. Other GSIs, which are peptidomimetic or small molecule γ-secretase inhibitors, LY411,575 or MRK003 respectively, used in our studies do not have aldehyde groups and have not been shown to inhibit calpain or other serine proteases (187).

Rizzo et al. used 1 µM LLNle, to inhibit Notch activation, and demonstrated growth inhibition in the cell lines tested including T47D-A18, MCF7, T47D-C42 and MDA-MB-231 cells (183). Curry et al. showed that treatment of Kaposi’s sarcoma cell lines and primary tumor cells with either of two different GSIs resulted in a G2/M growth arrest followed death via mitotic catastrophe and apoptosis (187, 189). Moreover,
treatment of established Kaposi’s sarcoma xenografts with LLNle led to growth inhibition and tumor regression (187). Chen et al. found that lung tumors treated with MRK003 had increased levels of apoptosis compared to the control (190). Finally, Konishi et al., using a lung cancer model showed that treatment with MRK003 decreased cell proliferation, inhibited Notch-3 signaling, and induced apoptosis (191). In another study, Qin et al. showed that LLNle had anti-neoplastic activities in melanoma (188). However, he later showed that LLNle, whose structure is similar to MG132 (a potent proteosomal inhibitor (reviewed in (192)), inhibited the proteosome under experimental conditions, which was responsible for the observed cell death (193).

Notch and Breast Cancer Stem Cells

Several studies have linked the Notch pathway to breast CSCs. Farnie et al. showed that recurrence (at 5 years) of DCIS was increased in patients whose samples stained positive for N\textsuperscript{IC} (194). They also found that mammosphere formation efficiency from DCIS samples was higher than in normal breast tissue. When treating mammospheres with an anti-Notch-4 antibody or a GSI (DAPT), the authors found a decrease in mammosphere formation efficiency, but did not see evidence of cell death under their experimental conditions (194).

Sansone et al. showed that mammospheres derived from MCF7 cells and primary ductal carcinoma samples expressed stem cell-associated genes such as Bmi-2, CD44, Oct-4 and CD133 (195). They reported that Notch-3 was critical to maintenance of mammospheres as MCF7 cells transduced with siNotch-3 showed a decrease in mammosphere size, which is consistent with inhibition of progenitor proliferation (not self-renewal). Previously, these authors had found Notch-3 along with
p66Shc (a mammalian longevity modulator) and Jag-1 promote self-renewal (as mammospheres) under hypoxic conditions. These authors pursued Notch-3 because its overexpression has been shown to cause tumors in mice and because the Notch pathway can interact with hypoxia inducing factor (HIF) to promote a stem-like phenotype (196, 197). Furthermore, Notch-3 was highly expressed in mammospheres derived from tumors as compared to normal tissue (128, 195, 198).

Clearly, there is ample evidence that Notch signaling plays an important role in breast cancer and the stem-like population. Breast cancer cells express higher levels of activated Notch and the ligand Jag-1 than normal breast tumor cells, which is associated with a poor prognosis. Therefore, targeting this pathway may have clinical relevance.

**Cell Death**

Cell death can occur in several different ways: necrosis, autophagy, mitotic catastrophe, and apoptosis. These will be briefly summarized below with the focus being on apoptosis.

Necrosis is an unregulated form of cell death characterized by swelling and rupturing of the cell membrane degeneration and release of organelles. It is often caused by trauma, infection, or inflammation. *In vivo* this results in the accumulation of cellular debris in the areas that can be detrimental to the health of the surrounding tissue. Necrosis also has an effect on ion transport and pH balance. The direct mechanism of action of necrosis is not clear (199).
Autophagy is a form of cell death in which cellular organelles are degraded by lysosomes. The morphology of cells undergoing autophagy includes blebbing of the cell membrane and an increase in autophagic vesicles. These vesicles, which form in the endoplasmic reticulum, contain organelles and cellular material and fuse with lysosomes, resulting in their degradation. On the molecular level, PI3K and target of rapamycin (mTOR) may be involved in autophagy. mTOR negatively regulates autophagosome formation. The role of autophagy in cell death is still not fully understood but dysregulated autophagy has been implicated in several diseases including cancer (199).

Mitotic catastrophe refers to cell death following aberrant mitosis. It is initiated when a cell arrests during replication and is unable to repair the damage. Often, the cells arrest during interphase and form multiple micronuclei, which are characteristic of mitotic catastrophe. Ultimately, the cells will die via apoptosis, non-apoptotic cell death, or will enter senescence.

Apoptosis (programmed cell death) is characterized by cell shrinkage, membrane blebbing, chromatin compaction, and DNA fragmentation that eventually leads to the phagocytosis and elimination of the cell (200). It can be divided into three stages. First, apoptosis is initiated by either a ligand (extrinsic pathway) or cellular stress such as ultraviolet light (intrinsic pathway); these will be discussed below. Following initiation, caspases are activated. Caspases are cysteine proteases that exist in an inactive form (procaspases) (reviewed in (201)). There are three families of caspases in humans: initiator (caspase -8, -9, and -10), executioner (caspase -3, -6, -7), and inflammatory (caspase-1,-4,-5, -11, and -12) caspases. The initiator caspases form a complex with
either death receptors or adaptor proteins and become activated by autoproteolytic cleavage, then are released as activated caspases. Caspase -8 and -10 will form a complex with the receptor and adaptor proteins called the death inducing signaling complex (DISC). Caspase-9 will form a complex with cytochrome c and an adaptor protein called Apaf-1 (apoptotic protease activating factor 1). Once activated, a “caspase cascade” occurs whereby the initiator caspases activate the executioner caspases. The executioner caspases then cleave downstream targets associated with cell structure and function, leading to cell death. Caspase-2 cleaves a pro-apoptotic protein Bid that promotes the release of cytochrome c from the mitochondria (202). Inflammatory caspases are mainly associated with cleaving interleukins, which are important in the inflammatory response, or activating caspases associated with the inflammatory response.

The Extrinsic Pathway of Apoptosis

The extrinsic or death receptor pathway involves several members of the tumor necrosis factor (TNF) receptor superfamily. Fas, TNF-R1, TRAIL-R1 and TRAIL-R2 are cell surface receptors containing a conserved death domain in the cytoplasmic tail. Alternative designations for these receptors and ligands are listed in Table 2. When engaged by the appropriate ligand, the receptor trimerizes and recruits death domain-containing adaptor proteins that interact with the death domain in the receptor’s cytoplasmic tail. These complexes of adaptor proteins are referred to as DISC.
Each receptor recruits slightly different adaptor proteins to the DISC. Fas, TRAIL-R1 and TRAIL-R2 interact with FADD while TNF-R1 interacts with TRADD. Once the DISC has formed, the death effector domain of FADD, for example, binds to procaspase 8 (reviewed in (203)). The high concentration of procaspase-8 in the DISC leads to autoproteolytic cleavage releasing active caspase 8. This will then cleave executioner caspases-3, -6, or -7 resulting in cleavage of downstream apoptosis-associated targets such as DNA repair enzymes, cytoskeletal proteins, and nuclear proteins leading to cell death (reviewed in (204)). The extrinsic apoptotic pathways are summarized in figure 6.

<table>
<thead>
<tr>
<th>Full name</th>
<th>Abbreviation</th>
<th>Alternate abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor necrosis factor receptor-1, -2</td>
<td>TNF-R1*, TNF-R2</td>
<td>TNFRSF1A*, TNFRSF1B, CD120a*, CD120b</td>
</tr>
<tr>
<td>Tumor necrosis factor receptor superfamily 6</td>
<td>TNFRSF6</td>
<td>Fas, CD95, APO-1</td>
</tr>
<tr>
<td>TNF-related apoptosis inducing ligand receptor -1, -2</td>
<td>TRAIL-R1*, TRAIL-R2</td>
<td>DR4*, DR5, CD261*, CD262</td>
</tr>
<tr>
<td>Tumor necrosis factor alpha</td>
<td>TNFα</td>
<td>TNFSF2</td>
</tr>
<tr>
<td>TNF-related apoptosis inducing ligand</td>
<td>TRAIL</td>
<td>TNFSF10, APO2L, CD253</td>
</tr>
<tr>
<td>Tumor necrosis factor (ligand) superfamily member 6</td>
<td>TNFRSF6L</td>
<td>FasL, CD95L</td>
</tr>
</tbody>
</table>

Table 2: Alternative names for extrinsic apoptotic pathway ligands and receptors. Where two receptors are present the asterisk (*) identifies the corresponding pairs of abbreviations. DR = death receptor, TNFRSF = tumor necrosis factor receptor superfamily APO = apoptosis antigen ligand

Other members of the TNF receptor superfamily contain TRAF interacting motifs in their cytoplasmic tails including TNF-R2, CD40, CD30, and RANK. Activation of these
receptors recruits TRAF family members resulting in activation of signal transduction pathways including JNK, p38, and NF-κB. These receptors may play a role in apoptosis, but are also involved in cellular proliferation and differentiation.

The Intrinsic Pathway of Apoptosis

The intrinsic or mitochondrial pathway of apoptosis is initiated by a genotoxic stress such as ultraviolet light or cytokine deprivation. This form of apoptosis involves the Bcl-2 family, which consists of both pro- and anti-apoptotic members. There are over 20 Bcl-2-related proteins with most of the anti-apoptotic proteins found in the mitochondria, endoplasmic reticulum or nuclear envelope while the pro-apoptotic members are often found in the cytosol.

In healthy cells, Bax and Bak, two pro-apoptotic Bcl-2 family members, remain as monomers in the cytosol or loosely associated with membranes (reviewed in (205)). When a death signal is received, they undergo a conformational change and translocate to the mitochondria. Then Bax and Bak oligomerize and form pores in the mitochondrial membrane through which cytochrome c is released. Once cytochrome c is released from the mitochondria, it binds to Apaf-1 and procaspase-9 via its caspase recruitment domain. Together, these proteins form the apoptosome and activate the executioner caspases -3, -6, or -7 (199, 206).

Bcl-XL, an anti-apoptotic Bcl-2 related protein, inhibits cell death by binding to Apaf-1 and preventing formation of the apoptosome (reviewed in (204)). In contrast, Bcl-2 prevents apoptosis by blocking activation of Bax or Bak (205). Other pro-apoptotic
proteins include Smac/Diablo (second mitochondrial derived activator caspase protein) and Omni/HtrA2, which antagonize the inhibitors of apoptosis (IAPs) that bind to and inhibit activated caspases. The IAP family including XIAP (BIRC4), c-IAP1 (BIRC2), and c-IAP2 (BIRC3), inhibit apoptosis by binding caspases and preventing their activity (reviewed in (206)). XIAP binds to the active site of caspase-3 and -7, and also inhibits caspase-9 by preventing its dimerization. c-IAP2 is an E3 ligase that ubiquitinates caspase-3 and -7, whereas, c-IAP1 has been implicated in the degradation of TRAF2 which is an adaptor protein that binds to TNF-R1 and is responsible for activation of the JNK pathway (206, 207). Taken together, the balance between pro- and anti-apoptotic proteins plays a key role in determining whether a cell undergoes apoptosis.

Although the intrinsic and extrinsic apoptotic pathways are distinct, there is crosstalk between the pathways, and they converge at activation of the effector caspases. Activation of caspase-8 by the extrinsic pathway can lead to the cleavage of Bid to t-Bid (truncated Bid). t-Bid localizes to the mitochondria and induces cytochrome c release by promoting the formation of Bax and Bak oligomers (208). Bid is also cleaved by caspase-2 (202). This caspase can be activated via TNF-R1 resulting in induction of the caspase cascade and apoptosis (reviewed in (209, 210)).

**Breast Cancer Chemotherapy and Apoptosis**

Breast cancer chemotherapy results in the apoptosis of tumor cells. Both malignant and benign breast tumor cells have been shown to express members of the extrinsic and intrinsic apoptotic pathway including TRAIL receptors and members of the Bcl2 family (211). Single nucleotide substitutions, which resulted in missense mutations in the death domain or regions flanking the death domain of TRAIL-R1 or TRAIL-R2,
have been identified in metastatic breast cancer (212). The authors tested the effects of these mutations in TRAIL-R1, and TRAIL-R2 by creating constructs with the mutations and transfecting them into HEK293 cells then testing for apoptosis. They observed a significant decrease in apoptosis in these cells (212). With respect to the intrinsic apoptotic pathway, MCF7 cells increase Bcl-2 expression in response to estrogen. When these ERα-positive cells are treated with an anti-estrogen, such as Fulvestrant, Bcl-2 expression is decreased. This decrease of Bcl-2 in this system results in greater sensitivity to the chemotherapeutic agent Adriamycin (213). Most chemotherapeutic agents utilize the intrinsic pathway to induce cell death (reviewed in (204)).
Figure 6: A Summary of the Extrinsic and Intrinsic Apoptotic Pathways. Upon ligand binding to one of the receptors (TNF-R1, Fas, or TRAIL-R1,-R2), a downstream recruitment of various adaptor proteins occurs. In the case of TNF-R1 TRADD, SODD, FADD, RIPK2, RAIDD are recruited. FADD will bind to procaspase 8/10 through its death effector domain, which will then be activated by autoproteolytic cleavage. Caspase 8 will then activate either caspase -3, -6, or -7 (the executioner caspases) or cleave a Bcl-2 proapoptotic protein Bid that will then translocates to the mitochondria and activate Bax and Bak. Caspase-2, which binds to RAIDD via its CARD domain, can also cleave Bid resulting in t-Bid. These will allow for the release of cytochrome c from the mitochondria, which will bind to Apaf-1, and recruit caspase-9 via its CARD domain. Caspase 9 will bind activate executioner caspases 3, 6, 7. Bcl2 is an anti-apoptosis protein that prevents the release of cytochrome c by interfering with the production of Bax and Bak channels. In addition, Bcl2 can be regulated by the JNK pathway, which can be activated through TNF-R1, and a MAPKKK protein ASK. ASK can also phosphorylate MKK7 which can then activate JNK. JNK has also been implicated in inhibition of Bcl2. XIAP is an inhibitor of apoptosis, which binds to and inactivates caspase-3. Smac/Diablo are pro-apoptotic proteins found in the mitochondria, which will inhibit XIAP and inhibitors of apoptosis. Abbreviations XIAP= X-inhibitor of apoptosis; TNF-R1 = Tumor necrosis factor receptor 1; CARD = Caspase recruitment domain; FADD= Fas associated with death domain; SODD = Silencer of Death Domains; TRADD= TNFRSF1A-associated via death domain; RAIDD = RIP-associated ICH1/CED3 homologous protein with death domain; RIPK2 = receptor interacting serine-threonsine kinase 2; MKK7 = mitogen activated protein kinase kinase 7; ASK = apoptosis signal regulating kinase; Apaf-1 = Apoptotic peptidase activating factor-1; JNK = c-Jun N-terminal kinase; TRAIL = TNF related apoptosis inducing ligand
Apoptosis in the Putative Cancer Stem Cells

Studies have recently begun to approach the question of which apoptotic pathways may be used by CSCs. For example, TRAIL-R1 has been shown to be upregulated in the SP compared to the non-SP in colon cancer cells (214). The SP cells were also much more sensitive to TRAIL killing than their counterparts (214). The CD133-positive subset of MCF7 cells was found to overexpress FLIP (FLICE like inhibitory protein), an anti-apoptotic protein that inhibits caspase-8 by binding to it and preventing DISC formation (215). This study also showed that XIAP was upregulated in the CD133-positive fraction of glioblastoma cells (216).

The intrinsic pathway has also been implicated in CSC apoptosis. In leukemia, elevated Bcl-2 levels were found to protect hematopoietic stem cells from chemotherapeutic agents (217). In glioma stem cells, the anti-apoptotic protein Mcl-1 was highly expressed leading to resistance to treatment with a Bcl-2 inhibitor (218). Finally, Bcl-2 and Bcl-X_L were overexpressed in the CD133-positive subset of glioblastoma cells compared to the CD133-negative population.

While most chemotherapeutic agents engage the intrinsic apoptotic pathway, they are not always effective which may be due to the presence of the stem-like population. This may be due to the high levels of ABC transporters found in stem-like and early progenitor cells that efflux various toxic insults including chemotherapeutic agents from the cells. Drugs triggering signaling through the death receptor pathway can be used in combination with chemotherapy in order to promote cell death in tumors and possibly CSCs. Ligands of the extrinsic pathway do not need to enter the cell in order to
initiate a response. Therefore, members of the extrinsic pathway are an excellent target in CSCs for treatment of cancers since they can kill cancer cells from the outside of the cell and avoid efflux by the ABC transporters.

**Apoptosis via the c-Jun N-terminal Kinase Pathway**

Members of the JNK pathway are part of the MAPK family of kinases. Cellular stresses, cytokines and chemotherapeutic drugs can activate the JNK pathway which consists of three JNK proteins (JNK-1, -2, and -3). In order to activate JNK, a MAP3K (mitogen activated kinase, kinase, kinase) such as ASK-1, (apoptosis signal-regulating kinase 1) MEKK-1, -4 (MAPK/ERK kinase kinase-1, -4), or MLK-3 (mixed lineage kinase) needs to phosphorylate a downstream MAP2K (mitogen activated kinase kinase) such as MKK4 or MKK7. One of these kinases then phosphorylates JNK on a threonine-proline-tyrosine motif in the active site (219). In order to enhance JNK activation, MKK7 and JNK can bind to JIP1 (JNK interacting protein 1), a scaffold protein which allows the kinases to phosphorylate each other in close proximity (220).

In a study done by Harkin et al., the breast cancer cell lines MDA-MB-435 and HCC1937 were engineered to stably express BRCA-1 then screened to identify genes that were upregulated after induction of BRCA-1. The authors showed elevated levels of GADD45 (a DNA damage response gene), which then activated the JNK pathway by binding to the MEKK-4 (MKK4) (221) and led to apoptosis in a p53-independent manner (222). In the breast cancer cell line MBR62, BRCA-1 led to the upregulation of GADD45 and the JNK/SAPK pathway, which led to apoptosis (222).
Thangaraju et al. showed the JNK pathway was activated in MCF7 cells following serum deprivation. Interestingly, Fas/FasL upregulation were observed in response to JNK activation, which indicates that JNK may be upstream of the Fas pathway (223). Similar results were shown in ovarian carcinoma cells treated with cisplatin, which led to phosphorylation of c-Jun, and transcription of Fas ligand. When the JNK pathway was blocked, lower levels of Fas ligand mRNA and apoptosis were observed (224). Koyuturk et al. treated cells with simvastatin (commonly used to treated high cholesterol but also implicated in reduction of cancer) to show MCF7 cells and MDA-MB-231 engaged JNK to induce apoptosis (225).

Activated Notch as an Inhibitor of the c-Jun N-terminal Kinase Pathway.

Previous studies have linked the JNK and Notch pathways. Kim et al. showed that presenilin-1 (a component of the γ-secretase complex) prevented the activation of the JNK pathway in HEK 293 cells after exposure to ultraviolet light (226). They speculated that this inhibition might be due to activated Notch. Later, they showed that the activated form of Notch-1 interacted with JIP-1 thereby preventing the activation of JNK (227).

p38 Mitogen Activated Protei Kinase and Apoptosis

The p38 MAPK pathway has also been implicated in apoptosis. The p38 pathway can be activated by stress or cytokines such TNF-α, heat shock and some chemotherapeutic agents (Paclitaxel, Vincristine and Vinblastine). This pathway involves the activation of a MAP3K such as ASK1, or p21 activated kinase, which then
phosphorylates a MAPK2K, MKK-3 or MKK-6 at threonine and tyrosine residues leading to the activation of p38 MAPK (228). Downstream targets of p38 include ATF-2, MEF-2 and CDC25 to name a few. GADD45 was also shown to be an upstream mediator of p38 in human keratinocytes (229). In GADD45 null mice, p38 expression was low compared to mice with normal levels of GADD45 (229). The p38 MAPK pathway has also been implicated in the apoptosis of ovarian carcinoma cells. Seidman et al. showed that high concentrations of Taxol (1 μM-10 μM) increased p38 levels within two hours, and they continued to be active for up to 24 hours in ovarian carcinoma cells (230). Inhibition of p38 with SB203580 partially protected ovarian carcinoma cells from Taxol induced apoptosis. The p38 MAPK pathway was also induced (as determined by Western blotting) in endothelial cells treated with TNFα. In addition, capase-8 activation and levels of tBid were decreased in the presence of p38-MAPK inhibitor. These data link the p38 MAPK pathway with TNF signaling in endothelial cells.
Breast Cancer Cell Lines: The ERα-positive, estrogen-responsive breast cancer cell lines, T47D-A18 and MCF7, were grown under estrogen-containing conditions (RPMI-1640, 10% fetal bovine serum (FBS), 1% nonessential amino acids, 2 mmol/l L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 6 µg/ml insulin). T47D-C42 cells, an ERα-negative, hormone-nonresponsive breast cancer cell line, was grown under estrogen-deprived conditions (phenol-red free RPMI-1640 media, 10% charcoal-dextran stripped FBS, 1% nonessential amino acids, 2 mmol/l L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 6 ng/ml insulin). The T47D-A18 and T47D-C42 cell lines were originally subcloned from the T47D line and characterized in the laboratory of Dr. V. Craig Jordan. They have been extensively used as a model of acquired resistance to endocrine therapy (231, 232). Both cell lines were the kind gift of Dr. Debra Tonetti (University of Illinois at Chicago, Chicago, IL). MDA-MB-231 breast cancer cells, an ERα-negative, PR-negative, HER2-negative (triple-negative), and claudin low cell line, was grown in IMEM media containing 5% FBS, 1% nonessential amino acids, 2 mmol/l L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. BT474 and SKBR3 breast cancer cells, which are ERα-positive and negative respectively and both overexpress Her2/neu, were grown in IMEM media containing...
10% FBS, 1% nonessential amino acids, 2 mmol/l L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin. MDA-MB-468 cells, an ERα-negative, PR-negative, HER2-negative (triple-negative) cell line was grown in DMEM-Ham's F12 (50:50), 10% FBS, 2 mmol/l L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. Table 2 summarizes some key characteristics of the cell lines used in these studies. LTK-Jag-1 cells, a mouse L cell line overexpressing Jag-1, was grown in DMEM containing 10% FBS, 2 mmol/l L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. Some characteristics of these cell lines are summarized in Table 3.

Primary Breast Cancer Cells From Pleural Effusions: Pleural effusions were collected from patients undergoing thoracentesis for metastatic breast cancer with informed consent (LUMC). Effusions were diluted 1:1 in sterile phosphate-buffered saline (PBS) (Hyclone), the cells pelleted by centrifugation, and resuspended in sterile PBS. For all protocols, PBS will refer to PBS without calcium and magnesium unless otherwise specified. The cells (25 ml) were layered on 15 ml Ficoll-Paque (GE Healthcare, Piscataway, New Jersey) density gradient. Cells were then collected from the interface following a 30-minute centrifugation at 500 x g at room temperature. The cells were washed three times with PBS, counted, and used in various experiments. Table 4 lists the pleural effusion samples that were obtained.
Notch Inhibitors: Where indicated, cells were treated with one of three structurally distinct GSIs: Z-Leu-Leu-Nle-CHO (LLNle; EMD Biosciences, San Diego, CA), LY-411,575 (N2-((2S)-2-(3,5-difluorophenyl)-2-hydroxyethanoyl)-N1-((7S)-5-methyl-6-oxo-6,7-dihydro-5H-dibenzo(b,d)azepin-7-yl)-L-alaninamide) (Dr. Todd Golde, Mayo Clinic, Jacksonville, FL) or MRK003 (Merck pharmaceuticals, Whitehouse station, New Jersey). LLNle is a tri-peptide GSI, LY411,575 is benzodiazepine peptidomimetic GSI, and

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Molecular Classification</th>
<th>ERα/PR/HER2 Status</th>
<th>p53 Status</th>
<th>Tumor Type</th>
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<tr>
<td>BT474</td>
<td>Luminal</td>
<td>ERα+/PR+/HER2+</td>
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<tr>
<td>MDA-MB-231</td>
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<td>Pleural effusion</td>
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<tr>
<td>MDA-MB-468</td>
<td>Basal</td>
<td>ERα-/PR-/HER2-</td>
<td>[+]</td>
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<tr>
<td>SKBR3</td>
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<td>ERα+/PR+/HER2+</td>
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<td>Pleural effusion</td>
</tr>
</tbody>
</table>

Table 3: A summary of breast cancer cell lines utilized in our studies. ERα = estrogen receptor alpha, PR = progesterone receptor. WT= wildtype, M= mutant. Brackets [ ] indicate that information was obtained from only the mRNA and not protein. Information obtained from Neve et al. (233).

<table>
<thead>
<tr>
<th>Primary samples</th>
<th>ER/PR/HER2 status**</th>
</tr>
</thead>
<tbody>
<tr>
<td>BC-PE1*</td>
<td>ERα+</td>
</tr>
<tr>
<td>BC-PE2</td>
<td>HER2-overexpressing</td>
</tr>
<tr>
<td>BC-PE3</td>
<td>ERα+</td>
</tr>
<tr>
<td>BC-PE4</td>
<td>ERα-/PR-/HER2- (triple negative)</td>
</tr>
<tr>
<td>BC-PE5</td>
<td>ERα+</td>
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<td>ERα+</td>
</tr>
<tr>
<td>BC-AS1</td>
<td>ERα+/PR+/HER2-</td>
</tr>
</tbody>
</table>

Table 4: A list of the primary samples obtained from the Cardinal Bernardin Cancer Center, LUMC. BC-PE = breast cancer pleural effusion sample, BC-AS = Breast cancer ascites sample. The asterisk (*) indicates that these two samples were obtained from the same patient. ** The ER/PR/Her2 status represents the phenotype of the original tumor.
MRK003 is a cyclic sulfonamide small molecule GSI (234). MRK003 is an experimental drug synthesized by the Merck company. It is the research equivalent of the clinical drug MRK0756 that is currently in clinical trials. MRK0756 is not appropriate for animal studies; therefore, the MRK003 compound is utilized in preclinical studies. The half-life of the LY411,575 has been reported as 2 hours in vivo (235). To maintain inhibition of Notch signaling in vitro, we routinely replenish this drug every 6 hours (187, 189). LLNle is more stable and is generally replenished every 48 hours; while MRK003 is stable for 96 hours in vitro. We replenished MRK003 every 4-5 days based on the experimental protocol. All three agents were suspended in dimethyl sulfoxide (DMSO). A specific Notch decoy protein, recombinant human Notch-1 Fc chimera, and a control protein, recombinant human IgG1 Fc protein, were purchased from R&D Systems (Minneapolis, MN). Both reagents were resuspended in mammosphere media. A web-based calculator (molbio.ru/eng/scripts/01-04.html) was used to determine the concentration of each protein in micromoles/ml from the molecular weight.

**Kinase inhibitors:** A variety of kinase inhibitors were utilized including: JNK inhibitor II (10 mM), PD 98059 (MEK I inhibitor) (75 mM), SB203580 (p38 Inhibitor) (30 mM), NF-κB activation inhibitor (1 mM), or AG490 (10 mM). Each inhibitor was resuspended in DMSO at the listed concentrations and stored at -20 °C. The inhibitors were used at 1X, 2X and 10X their published IC\(_{50}\) concentrations. These inhibitors were purchased from EMD Biosciences.

**Side Population Analysis:** T47D-A18 and T47D-C42 cells were loaded with Vybrant DyeCycle Violet (DCV, Invitrogen, Carlsbad, CA), an alternative to Hoechst 33342, as described (135). Briefly, cells were suspended at 1 x 10\(^6\) cells/ml in DMEM containing
10% FBS and 10 mM HEPES (N-2-Hydroxyethylpiperazine-N'-2-Ethanesulfonic Acid) then incubated for 90 minutes at 37 °C with 5 µl (25 µM) of DCV. To confirm the SP had been identified, one sample of cells was pretreated for 30 minutes with 50 µM – 100 µM verapamil hydrochloride, an inhibitor of ABC transporters, to block DCV efflux. Variability problems in terms of reducing the SP were occasionally encountered with verapamil treatment, which has been previously reported (236). The cells were placed on ice, propidium iodide (PI) (2.5 µg/ml) added, and the cells immediately analyzed on a FACS Aria flow cytometer (BD Biosciences) by the Loyola University FACS Core Facility. After exclusion of dead cells with PI (excitation wavelength 536 nm emission wavelength 617 nm), the SP was identified by exciting DCV using the 405 nm wavelength. DCV emits at two different wavelengths 450 nm and 670 nm. The dye when added at saturating conditions will bind to DNA and emit at 450 nm while dye that has not bound to DNA emits at 670 nm. Since the unbound dye can be effluxed from the cell a decrease in fluorescence is observed at the 670 nm wavelength. Therefore, the SP can be identified as the small subset of cells with relatively low fluorescence compared to the bulk cell population (non-SP cells).

**Mammosphere Formation:** Primary mammospheres were generated from single cells by seeding ultra-low attachment plates (Corning, Lowell, MA) with approximately 20,000 cells/ml in mammosphere media [mammary epithelial basal media (Lonza, Walkersville, MD), 2% B27 (Invitrogen), 20 ng/ml EGF (BD Biosciences), 20 ng/ml bFGF (BD Biosciences), 4 µg/ml heparin (Sigma-Aldrich, St. Louis, MO)] as described (237). A total of 60,000 cells (in a 3 ml volume) were added to each well in a 6-well ultralow attachment plate or 360,000 cells (in a 10 ml volume) to a 10 cm ultralow attachment plate.
To passage mammospheres, cells were collected by centrifugation, 200 x g for 5 minutes, incubated for 3-5 minutes in 0.125% trypsin, and then cell clusters disrupted by pipetting through a 200 µl pipet tip. Microscopic examination was used to ensure single cell suspensions (≥99%) were obtained, and cell viability determined using trypan blue exclusion (1:2) prior to plating at 1000 cells/cm² in mammosphere media on ultra-low attachment plates (237). These cells were allowed to form secondary mammospheres and these were used for analysis.

In experiments determining the effect of GSI on mammosphere formation, primary mammospheres were prepared then treated after 24 hours with GSI or DMSO as a control. Twenty-hours later (48 hours after the start of the experiment) the mammospheres were dissociated as stated above and secondary mammosphere cultures established in the presence of fresh GSI or DMSO. Forty-eight hours after establishment of secondary cultures, mammosphere formation was quantitated by counting the number of mammospheres in 10 random, high-powered fields (HPF) per experimental condition. Any group of cells containing more than 4 cells was considered a mammosphere. Where indicated, cells were retreated with GSI or DMSO at 96 hours after establishment of secondary cultures. In studies utilizing the specific Notch decoy protein, primary mammospheres were dissociated and secondary mammosphere cultures initiated in a 96-well ultralow attachment plate in the presence of recombinant human Notch-1 Fc chimera (3 µM) or recombinant human IgG1 Fc (3 µM) as a control. Mammosphere formation was evaluated 24 hours later.

**Immunostaining:** Mammospheres were collected, centrifuged at 200 x g for 5 minutes and suspended in 100 µl of PBS. Cytospin cell preparations were then prepared using a
Shandon Cytospin 2 centrifuge at 500 revolutions per minute for 5 minutes. Cytospins were fixed in 50:50 methanol:acetone for 10 minutes and immunostained using a highly sensitive avidin-biotin immunoperoxidase technique (Vectastain ABC kit, Vector Laboratories, Burlingame, CA) as described (238). After fixing, the cells were blocked in horse serum (1:200 dilution in PBS + 0.1% BSA) for 15-30 minutes. The primary antibodies used for these studies were purchased from Leica Microsystems (Bannockburn, IL) and were diluted in PBS + 0.1% BSA. These include cytokeratin 5 (1:100) for progenitor cells, epithelial specific antigen (ESA) (1:500), cytokeratin 18 (1:400) for luminal epithelial cells, CD10 (1:80) and cytokeratin 14 (1:20) for myoepithelial cells (237). Cells were incubated with the primary antibody for 1 hour at room temperature in a humidified chamber, and then the slides were washed in 0.01% FA Buffer (BD Biosciences) for 5 minutes. Next, the cells were incubated with a species-specific secondary antibody (1:200) for 30 minutes at room temperature in a humidified chamber. Again, slides were washed in FA Buffer for 5 minutes. The avidin: biotinylated enzyme complex “ABC” reagent was added (1:100 for each reagent), incubated in a humidified chamber for an additional 30 minutes at room temperature, and washed with FA buffer for 5 minutes. A substrate, 3,3′-diaminobenzidine (DAB) (Vector laboratories), was prepared following the manufacturer’s instructions and added for color development. After an 8 minute incubation with DAB, the slides were washed with FA Buffer and counterstained with hematoxylin for 1 minute and washed with water 2 times once for 1 minute then for 5 minutes. Slides were mounted with Aquamount (Lerner Laboratories, Pittsburg, PA) and coverglass (Fisher Scientific Hanover Park IL).

Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) Assay:

TUNEL staining was performed using the ApopTag peroxidase in situ apoptosis
detection kit (Millipore) following the manufacturer’s instructions. Mammospheres derived from T47D-A18 cells were treated with 20 µM of MRK003 or DMSO for 24 or 48 hours and used for cytospin cell preparations. The cells were fixed in 4% neutral buffered formalin for 10 minutes at room temperature, and post-fixed in cold ethanol:acetic acid (2:1) for 5 minutes at -20°C to permeabilize the cells. The slides were washed twice in PBS (5 minutes each), and then 50 µl microliters of equilibration buffer added to the slides for 5 minutes at room temperature. The buffer was removed and TdT enzyme was added followed by a 1-hour incubation at 37°C in a humidified chamber. The slides were then soaked in stop buffer agitated for 15 seconds followed by a 10-minute incubation. Three 1-minute washes in PBS followed. The anti-digoxigenin conjugate was applied, incubated with the cells for 30 minutes in a humidified chamber, followed by four washes with PBS (2 minutes each). DAB was used as the substrate, but in this experiment, the nickel reagent was added to give a black color that is more easily discernable. The slides were incubated with DAB for 5 minutes, washed 3 times in water (1 minute each), and cells counterstained with methyl green (Vector Laboratories). Methyl green was added directly to the slides for 5 minutes at 60 °C and rapidly washed with water 2 times. The cells were then dehydrated in preparation for permanent mounting with Cytoseal 60 (Richard Allen Scientific Kalamazoo, MI). A permanent mount was required as methyl green is water soluble and is therefore not compatible with an aqueous mounting media.

**CD44, CD24, ESA Staining:** Standard flow cytometry techniques were utilized for the identification of CD44⁺ and CD24⁻ cells. Briefly, cultured cells were trypsinized to form a single cell suspension and washed twice with FACS buffer (1% FA buffer (Difco), 0.1% sodium azide, 1% FBS). Next, 5 x 10⁵ cells were incubated with primary antibodies.
Following a 1-hour incubation on ice, the cells were washed twice with FACS buffer and centrifuged at 5 minutes at 200 x g to collect the cells. When unconjugated primary antibodies were used, the cells were then incubated for an additional hour on ice with conjugated, species-specific secondary antibodies directed against immunoglobulin. Following two washes with FACS buffer, the cells were fixed in 2% paraformaldehyde, pH 7.4. Each experiment included appropriate controls including unstained cells for setting the appropriate gates, and cells stained with each antibody individually (compensation controls). Compensation controls are required when analyzing samples stained with multiple antibodies to determine and remove the spectral overlaps between the fluors (239).

For dual staining experiments, samples were incubated with CD24 (ML5, mouse IgG2a, BD Pharmingen) antibody at 5 µg/ml and CD44 (A020, EMD Biosciences) antibody at a 1:1000 dilution. Secondary antibodies included an allophycocyanin (APC) - conjugated goat anti-rat Ig polyclonal antibody (BD Biosciences) used at a 1:20 dilution and an allophycocyanin-Cyanine 7 (APC-Cy 7) goat anti-mouse IgG2a antibody (Southern Biotech, Birmingham, Alabama) used at a 1:150 dilution (144). For triple staining experiments, a CD44-FITC (Clone 156-3C11) conjugated antibody (Cell Signaling Technologies, Beverly, MA) and an ESA (Clone HEA-125) –APC conjugated antibody (Miltenyi Biotech, Auburn, CA) (144) were both used at a 1:10 dilution. The CD24 antibody (FL-80) (Santa Cruz Biotechnology, Santa Cruz, CA) was used at a 1:50 dilution and was detected with a PE-conjugated goat anti-rabbit IgG secondary antibody (1:200, Invitrogen). Based on published studies, the triple staining experiments included a few modifications from the standard protocol such as a PBS buffer containing 0.1% BSA + 2 mM HEPES was used instead of FACS buffer for resuspension of the cells.
Because these studies used a mixture of conjugated and unconjugated antibodies, the CD44-FITC antibody was added during the 1-hour incubation with the secondary antibody detecting CD24. During the last 10 minutes of this incubation, Fc blocking buffer (1:10, Miltenyi Biotech) was added followed by the ESA antibody. Although most staining procedures use longer incubation times, the manufacturer recommends only a 10 minute incubation with the ESA-APC antibody.

**Aldehyde Dehydrogenase Activity (Aldefluor) Assay**: The Aldefluor assay (Stem Cell Technologies, Vancouver, British Columbia, Canada) was performed according to manufacturer’s instructions. Cells were washed with PBS and suspended at 1x10^6 cells/ml in assay buffer, and 5 µl of Aldefluor reagent was added to 1 ml of cells. Immediately, 500 µl of cells was transferred to a tube containing 5 µl of diethylaminobenzaldehyde (an ALDH inhibitor). Cells were placed in a 37°C water bath for 45 minutes then analyzed by flow cytometry using the FITC (488 nm wavelength) channel. By comparing the flow profiles of cells with and without the ALDH inhibitor, the ALDH-containing population can be readily identified.

**Quantitative Reverse Transcriptase Polymerase Chain Reaction (RT-qPCR)**: Total cellular RNA was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA) as directed by the manufacturer. Complementary DNA was synthesized by reverse transcription using 5.5 mM MgCl₂, 500 µM dNTP (deoxynucleotide triphosphate), 2.5 µM oligo dT and 2.5 µM random hexamers as a primer, 0.4 U/µL RNase inhibitor and 1.25 U/µL reverse transcriptase enzyme (Taqman Reverse Transcription kit, Applied Biosystems Foster City, CA). The reverse transcription reaction used 200 ng of RNA for each reaction. cDNA was synthesized by placing the reaction at 25°C for 10 minutes, 48°C for 30
minutes, and 95°C for 5 minutes. RT-qPCR was performed using an Applied Biosystems 7300 sequence detection system (Foster City, CA) with Quantitect SYBR Green PCR reagents and Quantitect Validated Primer Sets (Qiagen) following the manufacturer's instructions. The validated primer sets were designed to span exon-exon borders preventing amplification of genomic DNA, and to prevent amplification of non-specific PCR products and primer-dimers. To help prevent PCR contamination, all reactions contained dUTP and 0.025 U/µL uracil DNA-glycosylase to destroy previous amplified product (240). Negative control samples containing yeast RNA (250 ng/ml, Applied Biosystems, Foster City, CA) instead of cDNA was used to monitor PCR contamination in every experiment. The RT-qPCR program was set as follows: 50 °C for 2 minutes, 95 °C for 10 minutes, then 40 cycles of 95 °C for 15 seconds, 55 °C for 30 seconds, and 72 °C for 30 seconds. Additionally, a dissociation curve was run for each experiment. In all experiments, only one peak was observed in the dissociate curve indicating that non-specific amplification had not occurred. Amplification efficiencies for the primer sets were shown to be approximately equal using a validation experiment (Applied Biosystems, User Bulletin 2: Relative Quantitation of Gene Expression). Relative mRNA expression was calculated using the comparative method (described below) where expression of the target genes in each sample was normalized to β2-microglobulin expression (Applied Biosystems, User Bulletin 2: Relative Quantitation of Gene Expression).

The comparative method is used to compare mRNA expression levels between two samples enabling a fold-change to be calculated. The Applied Biosystems 7300 sequence detection system captures the fluorescent signal in each sample generated by binding of SYBR green to the double-stranded PCR product at the end of each PCR cycle. As the amount of SYBR green fluorescence is directly proportional to the amount
of PCR amplification, a plot of the data obtained at each cycle provides the necessary information to calculate relative mRNA expression. By setting a threshold value within the linear range of the amplification plot, but above the region where the non-amplified region is found, a \( C_T \) (cycle threshold) value can be calculated. This value is defined as the number of cycles required for the fluorescent signal to cross the threshold. \( C_T \) values are inversely proportional to the amount of target cDNA in the sample. The \( C_T \) values are normalized against the \( C_T \) values for \( \beta 2 \)-microglobulin to compensate for differences in the sample RNA (such as amount added or quality of the RNA that could affect amplification etc). This value is termed \( \Delta C_T \). The \( \Delta C_T \) value for the control sample is then subtracted from the \( \Delta C_T \) value of the test sample giving a \( \Delta \Delta C_T \) value. This value is then used to calculate the fold change using the equation \( 2^{(-\Delta \Delta C_T)} \) (Applied Biosystems, User Bulletin 2: Relative Quantitation of Gene Expression). A fold-change of 2 or higher is generally considered significant.

**Western Blotting:** Whole cell extracts were prepared from mammospheres and bulk cultured cells. Mammospheres were collected by centrifugation 200 x g for 5 minutes, washed one in ice-cold PBS and then lysed with 75 \( \mu \)L-100 \( \mu \)L ice-cold RIPA buffer (0.1% Sodium dodecyl sulfate (Sigma), 1% Noniodet P40 (Roche)) containing freshly added 0.2% sodium deoxycholate (Sigma). Immediately before protein extraction one-fourth tablet of Mini complete protease inhibitor cocktail was added to 2.5 ml of extraction buffer. Cell monolayers were treated similarly, with the exception that they were rinsed with cold PBS and lysed directly on the tissue culture plates. The plates were then scraped using a cell lifter (Corning) to collect the cells. The extracts were briefly sonicated (three pulses of 3-5 seconds each) using a Fisher Scientific Sonic Dismembrator Model 100 (setting 2), and incubated for one hour on ice. The samples
were then microcentrifuged for 20 minutes 16,000 x g at 4°C to pellet insoluble protein. Samples were stored at -80°C prior to analysis. Protein concentration was quantitated using a standard curve method using the Bio-Rad Protein assay reagent (1:5) (Bio-Rad, Hercules CA). Briefly, a standard curve was prepared ranging from 0-40 µg/ml, and then the protein samples were diluted 1:500 for the quantitation in the assay reagent. Quantification was done using the Omega Polar Star plate reader (BMG Labtech) at 595 nm.

Western blotting was performed using the Invitrogen XCell SureLock Mini-Cell Apparatus and the NuPAGE electrophoresis system. Samples were prepared by using 50 µg of total cellular protein and adding 4X NuPAGE LDS buffer and Milli-Q water for a 1X final concentration. Samples were run under non-reducing conditions. They were vortexed, and heated at 70°C for 10 minutes using an Eppendorf thermomixer 5436. The samples were loaded on a NuPAGE Novex 7% Tris-Acetate Gel, covered with NuPAGE Tris-Acetate SDS buffer, and run at 150 volts for approximately 1 hour. The proteins were then transferred to an Immobulon-P transfer membrane (Millipore). This polyvinylidifluoride membrane was first activated by immersion in methanol for 15 seconds, MilliQ-water for 30 seconds, and soaking in NuPAGE transfer buffer for 5 minutes. The transfer was performed using the XCell II Blot module with 1X NuPAGE transfer buffer for 2 hours at 30 volts. The membrane was then blocked with 5% non-fat milk (Bio-Rad) with Tris-buffered saline containing 0.1% Tween for 1 hour at room temperature followed by three washes in 1X Tris-buffered saline (25X TBS = 0.5M Tris-Base, 3.75 M Sodium Chloride, pH = 7.5) with 0.01% Tween (TBST) for 5 minutes each. The membrane was then incubated overnight at 4 °C with Hes-1 antibody (ab55265, Abcam) (2.5 µg/ml) in 5% milk in TBST. After three washes for 5 minutes each with
TBST, a secondary antibody (sheep anti-mouse IgG Horseradish Peroxidase linked whole antibody, (GE Healthcare, Piscataway, New Jersey) was added at a 1:3333 dilution and incubated in 5% in TBST, followed by three washes with TBST. The membrane was incubated with Pierce Supersignal Western Dura Extended Duration Substrate for 5 minutes and the results recorded using an Intelligent Dark Box LAS-3000 imager (Fujifilm Tokyo, Japan). β-tubulin (1:7500, Clone B512, Sigma) was used as a loading control. Following documentation of the Hes-1 results, the membrane was blocked again as described then incubated with the β-tubulin antibody diluted in 5% milk for 1 hour at room temperature. The remaining protocol was followed as stated above.

**Luciferase Assays:** The Hey-1 (also known as HesR1, HERP2, HRT1) luciferase reporter construct was the gift of Dr. M. Gessler (University of Wuerzburg, Wuerzburg, Germany). Approximately 3 kb of the promoter region (-2839 to +87) of Hey-1 was inserted in front of the luciferase gene in the promoterless vector, pLuc. T47D-A18 and T47D-C42 cells were plated at 2.5 x 10^5 cells/ml and 1x10^6 cells/ml respectively in a 96 well plate (Corning) in standard media without penicillin-streptomycin. The cells were allowed to attach and grow overnight (T47D-A18) or for 48 hours (T47D-C42) to achieve approximately 80% confluence. Transfection reactions were prepared using Lipofectamine 2000 (Invitrogen) so that each reaction contained ample reagent to perform the experiment in quadruplicate. Briefly, 1.25 μg (0.25 μg/well) Hey1-luciferase plasmid was added to 125 μl Opti-MEM media (Invitrogen). Next, 6.25 μl of Lipofectamine 2000 was added to a separate tube containing 125 μl of Opti-MEM media. Following a 5-minute incubation at room temperature, the solutions were mixed together and incubated an additional 20 minutes. Fifty microliters of DNA-Lipofectamine solution
was then added to the cells. Transfected cells were allowed to recover overnight prior to removal of the transfection media and subsequent treatments. In these assays, the cells were treated with a GSI or DMSO as a control for 24 hours prior to measuring luciferase activity using the Steady-Glo Luciferase Assay System (Promega, Madison WI) and a Turner Biosystems Veritas microplate luminometer. Following removal of the media, 25 µl PBS and 25 µl of Steady Glo reagent were added to each well, and the plate incubated for 5 minutes to ensure complete cell lysis. The samples were then transferred to a 96-well white (Lumitrac) microplate (Bioexpress, Kaysville UT) and read on the Veritas microplate luminometer. Transfection efficiency was determined by transfecting additional cells that were not treated with a plasmid expressing green fluorescent protein (GFP), percent transfection efficiency was determined by flow cytometry, and the average value of used to normalize the luciferase data as previously described (238). Previous studies from our lab have shown that co-transfection with firefly luciferase and renilla luciferase constructs resulted in serious problems with promoter crosstalk resulting in uninterpretable data. This is due to the presence of cryptic promoter elements in the backbone of the luciferase vector that can alter luciferase gene expression under certain conditions. Thus, we were unable to use a dual luciferase assay to determine both transfection efficiency and Notch-dependent luciferase activity in the same sample.

Determining Notch Activation Using the Notch Decoy: T47D-A18 cells were transfected with the Hey-1 luciferase reporter construct in 96 well plates as stated above. The following day, LTK cells overexpressing Jag-1 (LTK-Jag-1) were scraped from plates, counted and resuspended at 1x10^6 cells/ml in T47D-A18 media without penicillin-streptomycin. Fifty microliters of the LTK-Jag-1 cell suspension (or 5 x 10^4 total cells)
was incubated with 3 μM of the recombinant human Notch1-Fc protein or 3 μM recombinant human IgG1 Fc protein for 15 minutes, and then added to the transfected T47D-A18 cells. The cells were co-cultured for 24 hours prior to determining luciferase activity as described above.

**Determining Notch activity after transfection of an siNotch-1 plasmid:** T47D-A18 cells were plated in 10 cm tissue culture plates (Corning) so that they were 70-80% confluent (2.5x10^6 cells/plate) at the time of transfection. They were transfected with 1 μg of PLVTHM-siNotch-1, which also contained a GFP coding sequence using 150 μl of Lipofectamine 2000 as described above. Cells were passaged the following day. In order to achieve high levels of GFP expression cells were analyzed by flow cytometry 48 hours after transfection. First, cells were trypsinized and collected by centrifugation 200 x g for 5 minutes then resuspended in mammosphere media at 4x10^7 cells/ml and FACS sorted into GFP-positive and GFP-negative cells. These cells were grown as mammospheres under standard conditions.

**Proliferation Assay:** Mammosphere cultures were established in Corning ultra-low attachment 96 well plates by plating 3,200 cells/well (quadruplicate wells for each test condition) in the presence or absence of Notch inhibitors (GSIs or Notch decoy) or appropriate control reagents as stated above. After 48 hours, an MTT based assay, MTS, (3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium) was performed using the CellTiter96 AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI). To quantitate proliferation 20 μl of reagent was added directly to 100 μl of cells/media in culture of media. Absorbance was measured every hour for 4 hours on an Omega Polar Star plate reader (BMG Labtech) at 490 nm.
Background color development was calculated using wells containing media and CellTiter 96 Aqueous One Solution without cells.

**Colony Formation Assay:** Primary mammosphere cultures were established and treated after 24 hours with MRK003 (10-20 µM). After an additional 24 hours, mammospheres were dissociated and used in the colony forming assays. A 24 well plate (Corning) was prepared by placing 500 µl of 0.5% Noble agar (BD Biosciences) in mammosphere media in each well and allowing the agar to solidify for 30 minutes. Single cells from the dissociated mammospheres were counted using a hemacytometer and resuspended to 1.5 x 10^4 cells/ml. One milliliter of the cell suspension was added to 2 ml of warm 0.5% Noble agar in mammosphere media in the presence or absence of MRK003 (10-20 µM) or DMSO as a control. Five-hundred microliters of cell-agar suspension (2,500 cells/well) was placed on top of the agar-base in the 24 well plate. Plates were incubated at 37°C in 5% CO₂ and were examined every other day for up to 28 days. Every five days, 500 µl of fresh media with or without MRK003 or DMSO was added on top of the agar. Photographs were taken of 10 random high power fields every seven days to quantitate the results.

**Annexin V / Propidium Iodide Staining:** The ApoScreen Annexin V Apoptosis (Beckman Coulter, Brea, CA) kit was used to confirm MRK003 induced apoptosis. Mammospheres from T47D-A18 and SKBR3 cell lines were treated with either DMSO or MRK003 (20 µM) for 4 days and then collected and dissociated as already described. The cells were resuspended in 1X binding buffer, and 10 µl of Annexin V added to the samples. The samples were incubated for 15 minutes on ice. Two-hundred microliters of binding buffer was then added followed by 10 µl of PI. Each experiment included appropriate controls.
including unstained cells for setting the appropriate gates, and cells stained with only Annexin V or PI for compensation controls.

**Human Apoptosis RT² Profiler PCR Array:** The human apoptosis RT² Profiler PCR array (SABiosciences) was performed using manufacturer’s instructions. Because the primers used in this PCR array do not span exon-exon junctions, contamination of the RNA preparation with genomic DNA is a potential issue. Therefore, the RNA samples were treated with DNAase (Qiagen) for 10 minutes as described in the RNeasy Mini Kit protocol. In addition, the PCR array includes controls that evaluate genomic DNA contamination. The RT² First Strand Kit was used to synthesize cDNA for these studies. RNA (1 µg) was treated to eliminate genomic DNA and then cDNA prepared following the manufacturer's instructions. The RT² Real-Time SYBR Green/Rox PCR master mix was used for the RT-qPCR steps. SYBR Green mixture was prepared by adding diluted 2X SA Biosciences RT² qPCR Master Mix containing cDNA, and 25 µL of this mixture was added to the RT² Profiler PCR plate provided (which contained primers). Enough mixture was prepared for two plates (DMSO and MRK treated cells) and the plates were set up simultaneously to eliminate the potential differences that could confound interpretation of the assay. The samples were analyzed by Applied Biosystems 7300 sequence detection system using the following program 95 °C for 10 minutes followed by 40 cycles of 95° C for 15 seconds and 60°C for 1 minute. The data was analyzed by the comparative method using the SABiosciences web based data analysis software.

**Statistical Analysis:** Statistical analysis was performed using a Student’s t-test when comparing two populations. A p-value of less than 0.05 was considered significant.
CHAPTER 3
RESULTS

Hypothesis: The putative breast CSC contains elevated levels of activated Notch and depends on Notch signaling for self-renewal, survival, and/or proliferation. Notch inhibitors may adversely affect this population.

Specific Aim
Determine the effects of Notch inhibitors on the putative CSC, specifically on self-renewal, proliferation, and survival.

Identification of Putative Cancer Stem Cell
Four different methodologies are currently utilized to identify and isolate / enrich stem cells: side population (SP) (127), mammosphere formation (128), phenotypic markers (129, 130, 145), and aldehyde dehydrogenase-1 (ALDH-1) activity (131). We have attempted all four techniques in these studies, and determined that each had its own advantages and disadvantages.

Side Population
We began our studies by isolating the SP from two cell lines, T47D-A18 and T47D-C42. These subclones of the T47D cell line were chosen because they represent
two of the most common subgroups of breast cancer, ERα-positive (T47D-A18) and ERα-negative (T47D-C42). Moreover, because the cell lines possess the same genetic background, we hypothesized that we might be able to identify overt differences between ERα-positive and ERα-negative stem-like cells, with respect to Notch signaling.

Traditionally, Hoescht 33342 has been the dye used in SP analysis; however, we were unable to use it, as we could not obtain adequate fluorescence to discern the SP. This is because an ultraviolet laser is required to excite/detect this dye, and we do not have one in our core facility (LUMC). As an alternative, we used Vybrant DyeCycle Violet (DCV) (Invitrogen), a vital dye detected with a violet laser that has been effectively utilized in SP analysis (135). T47D-A18 or T47D-C42 cells were incubated at 37°C with DCV to allow uptake of the dye. The cells were placed on ice, PI added, and then analyzed by flow cytometry. Because stem cells should effectively efflux the dye due to elevated ABC transporter expression, they are less fluorescent than the bulk population and are readily identified in regions of low fluorescence. To confirm that the SP was correctly localized, control cell samples were pretreated with verapamil hydrochloride (50 µM-100 µM) to block ABC transporter activity. Following treatment with verapamil hydrochloride, the stem-like cells cannot efflux the dye and maintain a high level of fluorescence. Therefore, the SP will essentially “disappear” on the dotplot used for analysis. Figure 7 shows representative data from the two cell lines. Untreated cells contained on average 1.4% ± 0.1% SP for T47D-A18 and 1.2% ± 0.2% SP for T47D-C42 cells. As expected, verapamil hydrochloride treatment reduced the SP to 0.55% ± 0.1% (T47D-A18) and 0.6 ± 0.2% (T47D-C42). Similar experiments were performed in MDA-MB-231 and MCF7 cell lines, but we could not reproducibly identify a SP. Similarly, Patrawala et al. tested several breast cancer cell lines and reported no detectable SP in
MDA-MB-231 cells and only a 0.2% SP in MCF7 cells (137).

While useful for certain applications, the SP has several disadvantages. First, as mentioned above, a SP cannot be reproducibly isolated from all cell lines or cell samples despite the detection of stem-like cells via alternative methods. Second, the SP is considered stem cell enriched, but is not a pure population. The SP is contiguous with the non-SP (Figure 7), and it is not possible to completely separate the two populations. Moreover, any cell with low fluorescence, regardless of the reason, will fall into the SP

Figure 7: The side population (SP) can be identified in T47D-A18 and T47D-C42 cells. Representative dot plots showing the SP in T47D-A18 and T47D-C42 breast cancer cell lines. As anticipated, the SP consisted of <2% of the bulk cell population and could be effectively reduced by pre-treatment with the ABC transporter inhibitor, verapamil (p<0.01). Note that the SP fraction is contiguous with the non-SP cellular fraction. Therefore, they represent a subset of cells enriched for stem-like cells, but are not a pure population. Representative data, including the percentage of SP cells, are shown. At least 6 independent experiments were performed and averaged data from the combined experiments is listed in the text.
region. Given these issues, we utilized SP for only our initial studies. During the course of this work, new methods were described and validated, and we moved to these newer, more widely accepted methods for the remainder of the studies.

**Mammosphere Formation**

Sphere formation assays are the primary means of maintaining stem cells in culture. This technique involves culturing bulk tumor cells on ultralow attachment plates in specialized serum free media. Since most differentiated cells require attachment to survive, these non-adherent culture conditions select for the stem-like and early progenitor cells. Initial studies demonstrated that mammosphere formation is a measure of stem cell self-renewal, while the size of the mammospheres is a measure of progenitor proliferation (128, 241, 242). Dontu et al. found that primary mammosphere cultures prepared from normal human breast tissue were enriched for progenitor cells, but secondary and later mammosphere cultures consisted almost entirely of early progenitor cells (237). They calculated that each mammosphere was composed of 1-2 stem cells surrounded by bipotent and tripotent progenitors. Based on these studies, we primarily used secondary mammospheres for our experiments.

Initial experiments used immunostaining of cytospin cell preparations to confirm the mammospheres had a similar cellular composition to those described in the literature. We used well-known markers for mammary basal cells (CK-5), luminal cells (CK-18, ESA) and myoepithelial cells (CK-14, CD10). Mammospheres derived from T47D-A18 cells were found to contain cells that were CK-5-positive (26%), CK-18-positive (95%), ESA-positive (95%), and CK-14-positive (20%) (Figure 8). Less than 10% of the cells were faintly CD10-positive (Figure 8). While staining for T47D-A18 cells
has not been previously reported, these results are consistent with those published by
Dontu et al. and Farnie et al. (128, 194). Dontu et al. showed that mammospheres
derived from reduction mammoplasties were about 50% positive for ESA and few cells
stained positive for CK-5. Farnie et al. showed that 85% of cells in mammospheres
derived from DCIS were positive for ESA and about 30% were CK-14 positive, which is
consistent with Dontu et al. who showed 20% of cells were positive for CK-14 (128, 194).
From these results, we concluded mammospheres prepared under our experimental
conditions are similar to those from other laboratories. Differences may be due to the
different samples that were tested. As a separate approach, the SP and non-SP cells
from T47D-A18 cells were collected and used to establish mammosphere cultures.
Spheres only formed in cultures derived from SP cells but not non-SP cells (data not
shown). This is consistent with previously reports (128). Since these early studies, we
have successfully prepared mammospheres from numerous cell lines including T47D-
C42, MDA-MB-231, SKBR3, BT474 and MCF7 cells, and four out of six primary samples
from patients with drug resistant breast cancer pleural effusions.
Breast cancer stem cells were originally identified by Al Hajj et al. based on their $CD44^+CD24^{low/-}\lineage ESA^+$ phenotype (129). We used a similar strategy to identify CSCs in established cell lines. There was little to no evidence of a $CD44^+CD24^{low/-}$ population in T47D-A18 (0.7% ± 1.3) or T47D-C42 cells (0% ± 0.08). Even though the T47D-A18 cells did, on average, show a reasonable $CD44^+CD24^{low/-}$ population, there was significant variability between experiments as indicated by the standard error, making it impossible to use this method to collect stem-like cells from experiments.
Sheridan et al. also reported that the parental T47D cell line had 0% CD44⁺CD24<sup>low/-</sup> cells (144). In contrast, MDA-MB-231 cells consistently showed a vast majority of CD44⁺CD24<sup>low/-</sup> cells (96.1% ± 2.2). Similarly, published studies have also shown over 90% of MDA-MB-231 cells are CD44⁺CD24<sup>low/-</sup> (144, 145). Further studies by Fillmore and Kuperwasser found the tumorigenic subset of MDA-MB-231 cells could only be accurately identified if the cells were also evaluated for ESA. They reported 2.5% of these cells were CD44⁺CD24<sup>low/-</sup> ESA⁺ (145). Likewise, we found 5.5% ± 1.7 of MDA-MB-231 cells were CD44⁺CD24<sup>low/-</sup> ESA⁺ under our experimental conditions.

**Aldehyde Dehydrogenase-1 Activity (Aldefluor)**

The Aldefluor kit can be used to identify cells with elevated ALDH-1 activity that can then be identified and isolated by flow cytometry. We examined a variety of cell lines but found little to no evidence of an ALDH-positive population (T47D-A18 (0.33% ± 0.18), T47D-C42 (0.133% ± 0.08) and MDA-MB-231 cells (0.0% ± 0.08), even using cell lines that were previously reported to have this population (243). We contacted Dr. Gabriela Dontu, an author on the initial report, who stated that the FACS-Aria flow cytometer, which is available in our core facility, was not useful for these assays using cell lines (personal communication). The level of fluorescence in the ALDH-positive population in cell lines is substantially less than primary samples necessitating the use of a powerful, 200 mW argon laser. As the FACS-Aria has only a 40 mW laser, we were unable to use this technique for the majority of our experiments. However, we did find a distinct ALDH-positive population when using primary tumor cells from metastatic breast cancer pleural effusion samples.
Taken together, we successfully identified the breast cancer stem-like population using all four methodologies currently utilized by working with breast CSCs. However, significant limitations to each method were found based on the technique itself, the use of established cell lines, or lack of access to necessary equipment. As a primary goal of our studies was to compare and contrast results between tumor cells derived from different subtypes of breast cancer, mammosphere formation became the primary methodology used in the studies. Only mammosphere formation consistently enriched for the putative CSC in a majority of cultured cell lines and patient samples. This data could then be extended using other methodologies to identify and isolated the stem-like cells.

Notch Expression and Activation in the Putative Breast Cancer Stem Cell Population

Notch Activation/Expression in Side Population versus Non-Side Population

We began these studies using RT-qPCR to evaluate mRNA expression of Notch pathway related genes. First, Notch expression and activation were examined in SP and non-SP cells isolated from T47D-A18 and T47D-C42 cell lines. Bulk cultured cells were labeled with DCV and the SP and non-SP cells collected using FACS. Total RNA was extracted and cDNA synthesized. RT-qPCR was used to determine relative expression of Notch-1, Notch-4 and two downstream targets of Notch activation, Hes-1 and Hey-1. This is important because Notch expression does not indicate activation. Therefore, expression of downstream targets of Notch are commonly used as surrogate markers of activation.
The results showed a significant increase in expression of Notch-1, Notch-4, Hes-1, and Hey-1 mRNA ($p<0.05$) in SP derived from T47D-C42 cells compared to non-SP cells (Figure 9). In contrast, T47D-A18 cells showed a consistent increase in expression of these Notch-related genes, but the differences were not statistically significant (Figure 9). In both cell lines, no significant difference was observed in expression of Hey-L, Hes-5 or Hey-2 mRNA (data not shown), between SP and non-SP cells.

**Notch Expression/Activation in Mammospheres versus Bulk Cells**

To confirm and extend the results, we examined expression of an expanded set of Notch-related genes in mammospheres and bulk cultured (monolayer) cells from breast cancer cell lines (T47D-A18, T47D-C42, MCF7, MDA-MB-231, MDA-MB468, SKBR3, and BT474) as well as a primary tumor sample from a breast cancer pleural effusion (termed BC-PE6). To confirm the stem-like nature of the mammospheres, Nanog expression was evaluated. This transcription factor is well recognized as an
important mediator of stem cell pluripotency and self-renewal (244).

Interestingly, Hes-1 mRNA expression was significantly increased in mammospheres compared to bulk cells in all tested samples (Figure 10, p<0.05) clearly documenting elevated Notch activation in the stem-like and progenitor population in breast cancer. Other Notch target genes were also elevated in some, but not all samples (Figure 10). With respect to Notch receptors and ligands, the results showed at least one receptor and one ligand were elevated in mammospheres compared to bulk cells in each sample, although the actual receptor and ligand involved varied. Finally, Nanog was significantly upregulated in most of the tested samples, indicating mammospheres contained cells with a stem-like phenotype. Nanog was not increased in MDA-MB-231 or MDA-MB-468 mammospheres, which may be related to the phenotype of these culture adapted cell lines (Figure 10). The fact that spheres from these cell lines did not demonstrate increased Nanog expression does not mean that they are not stem-like-cells as other stem cell related markers such as Oct4 or Sox2 may be elevated.
Figure 10: Various Notch ligands, receptors and targets are upregulated in mammospheres versus bulk cells in breast cancer cell lines and a pleural effusion sample. Relative expression of Notch ligands, receptors and downstream targets of Notch activation (several members of the Hes and Hey family of genes and Delta), from seven breast cancer cell lines and one primary breast cancer pleural effusion sample (BC-PE6) as determined by RT-qPCR. mRNA expression in bulk cultured cells was set at 1.0 and relative mRNA expression in mammospheres calculated using the comparative method. The results represent combined data (average ± SEM) from three independent experiments where each qPCR assay was performed in triplicate. Statistical analysis was performed using a Student’s t-test where p<0.05 was considered significant. For the primary breast cancer pleural effusion sample, data from a single experiment performed in triplicate are shown, where the error bars represent the standard deviation within the experiment.
The results were confirmed at the protein level using Western blot analysis. As a large number of mammosphere cultures are required to isolated sufficient protein for Western blot, we limited our studies to expression of Hes-1 because it is a surrogate marker for Notch activation and was elevated in all mammosphere samples at the mRNA level. Figure 11 shows a representative Western blot analyzing proteins isolated from bulk and mammosphere cultures from four different cell lines. In each case, mammospheres expressed significantly more Hes-1 protein compared to bulk culture cells.

![Western blot analysis](image)

**Figure 11:** Western blot analysis for Hes-1 expression in mammospheres and bulk tumor cells from representative cell lines. The fold increase in Hes-1 expression in mammospheres compared to bulk tumor cells was calculated using densitometry and is presented at the bottom of the figure. Data are representative of 3 independent experiments showing similar results. B: bulk tumor cells; MS: mammospheres

Together, these data demonstrate that the Notch pathway is preferentially expressed and activated in the stem-like and progenitor cells derived from numerous breast cancer cell lines and a primary tumor sample. Moreover, the results were relatively consistent between analysis of mammosphere-derived stem-like cells or SP cells. We attempted to examine Notch expression and activation in the Aldefluor-positive versus Aldefluor-negative population isolated from a primary tumor sample (BC-PE3). Although we found Hes-1 (2.1-fold increase) and Nanog (2.7-fold increase) were
increased in the Aldefluor-positive population compared to the Aldefluor-negative subset, we did not obtain enough RNA to examine the full panel of Notch related genes. Unfortunately, we were unable to examine additional primary specimens as we could not obtain more patient material.

**The Effects of Notch Inhibition on the Putative Cancer Stem Cell Population**

Given the elevated levels of Notch expression and activation consistently found in the breast cancer stem-like population, we examined the effects of Notch inhibition on the cells. Initial studies used three structurally distinct GSIs to inhibit Notch signaling: LLNle, LY-411,575 and MRK003. We began our work with LLNle, a tripeptide, aldehyde-containing GSI, because of its potent inhibition of γ-secretase activity and its availability from commercial sources. However, LLNle has been shown to inhibit serine proteases when used at concentrations over 5-10 µM in breast cancer cell lines (Dr. L. Miele, unpublished data). While we used this GSI at concentrations (0.5-1 µM) well below this range, we confirmed the data with two additional GSIs, LY-411,575 and MRK003 (174, 234). These GSIs do not contain the aldehyde group responsible for protease inhibition and this effect was not reported for these two GSIs.

**Measuring Notch Activity in the Presence of Gamma-Secretase Inhibitors**

To determine the optimal concentration of each GSI for our experiments, we performed a luciferase reporter assay. We selected a pGL3 basic-based reporter construct where luciferase gene expression was driven by the 3-kb promoter region of the Hey-1 gene. T47D-C42 and T47-A18 cells were transfected with the plasmid using Lipofectamine 2000, treated with increasing GSI concentrations, and luciferase expression evaluated after 24 hours. Figure 12 shows a significant reduction in
luciferase expression in GSI-treated cells compared to control (DMSO)-treated cells. Specifically, luciferase activity was decreased by 6.7-fold for 0.5 µM LLNle, 3.0-fold with 25 µM LY-411,575, and 5.6-fold with 10 µM MRK003 (Figure 12).

![Figure 12: Gamma-Secretase-Inhibitors decrease Notch activation. (A). GSIs effectively block Notch activation as determined by a Notch-responsive luciferase reporter construct. Although baseline Notch activation was modest, a significant reduction in luciferase activity was noted. Results represent combined data (average ± SEM) from 2 experiments performed in triplicate. Similar results were noted for T47D-A18. LLNle = Z-Leucine-Leucine-Norleucine-CHO, LY = LY411,575, MRK = MRK003, RLU= relative luciferase units. (B). MRK003 significantly blocked Notch activation in treated mammospheres compared to control-treated spheres as determined by Hes-1 mRNA expression. Results are representative of two independent experiments performed in triplicate. Error bars represent SEM between replicates.]

We confirmed the downregulation of Notch activity following MRK003 treatment using RT-qPCR for Hes-1 mRNA. Mammospheres derived from T47D-A18 cells and treated for 24 hours with 20 µM MRK003 showed a 16-fold decrease in Hes-1 mRNA expression compared to DMSO-treated controls (Figure 12B).
Gamma Secretase Inhibitors Decrease the Side Population

We began these studies by examining the effect of GSIs on SP analysis. Bulk T47D-A18 and T47D-C42 cells were treated with one of the three GSIs and after 24 hours, the cells were loaded with DCV with or without verapamil treatment and analyzed by flow cytometry. Verapamil treated cells were used to confirm the correct cell population had been identified on flow cytometry. Figure 13 shows that all three GSIs significantly decreased the SP compared to the DMSO control-treated cells. This data indicates Notch signaling may be required for maintenance of the SP.

Gamma Secretase Inhibitors Prevent Mammosphere Formation

While informative, SP analysis could only be performed with a limited number of cell lines. Therefore, we turned to mammosphere formation assays as our primary method for examining the effects of GSIs on the putative CSC. Mammosphere cultures were established from various breast cancer cell lines and primary patient samples.
Once small spheres or cell clusters formed, the cultures were treated overnight with GSI, the spheres dissociated, and new mammosphere cultures established in the presence or absence of fresh drug. After two days, small spheres were evident in DMSO-treated samples (Figure 14A), while treatment with any of the GSIs revealed primarily single cells (Figure 14B, and data not shown).

Treatment of the stem-like and progenitor cells from T47D-A18 and T47D-C42 cells with LLNle or LY-411,575 initially blocked mammosphere formation (Figure 14B), but at 5 days after treatment, cell clusters were clearly re-forming in the GSI-treated samples indicating a reversible or temporary growth arrest (data not shown). In contrast, T47D-A18 and T47D-C42 derived mammospheres treated with MRK003 remained as single cells until 6 - 7 days after treatment when the cultures appeared to contain primarily debris and a condensed or fragmented cellular morphology (Figure 14C). No evidence of sphere formation was found in the MRK003-treated cultures even after continued culture for an extended period of time (12 days), or addition of fresh growth media (data not shown). It should be noted that while 10 µM or 20 µM of MRK003 were generally used, similar results were obtained with 5 µM MRK003; however, it took longer for cells to reach the phenotype seen in figure 14C (approximately 10 days) (data not shown).

We extended our studies using MRK003 GSI to additional breast cancer cell lines MDA-MB-231, MCF7, SKBR3, BT474, and six primary breast cancer pleural effusion samples (Figure 14D-G). Only four of the six primary samples formed mammospheres in culture making it impossible to evaluate the other two. Strikingly, in each breast cancer cell line and the four patient samples, MRK003 abolished
mammosphere formation resulting in cultures of fragmented, condensed cells and debris after 7 days (figure 14C and data not shown). The results were similar for ERα-positive (T47D-A18, MCF7), ERα-negative (MDA-MB-231, T47D-C42, MDA-MB-468), and Her2/neu-positive (SKBR3, BT474) cell lines as well as 4 of 6 patient samples (3 recurrent, ERα-positive and one triple negative pleural effusions) (Figure 14D-G).

Figure 14: Gamma-Secretase-Inhibitors prevent mammosphere formation in various cell lines and breast cancer pleural effusion samples. Secondary T47D-A18 mammosphere cultures were treated with 20 µM MRK003 or DMSO, and were observed for 7 days. (A) DMSO-treatment, 48 hours (insert shows a large mammosphere at day 7) (B) MRK003, 48 hours (C) MRK003, 7 days. Cultures were treated with the lowest GSI concentrations shown to effectively block Notch signaling in reporter assays (LLNle: 0.5 µM, LY-411,575: 25-50 µM, MRK003: 10-20 µM). Quantitation was performed by counting sphere-like structures. (D). Representative pictures showing mammospheres from different cell lines treated with 10 µM MRK003 and pictures taken on day 6. (E). Counting sphere-like structures in T47D-A18 and T47D-C42 after treatment with one of three GSIs. (F). Counting sphere like structures treated with MRK003 in various cell lines (MCF7, MDA-MB-231, BT474 and SKBR3 cells). (G). Treatment of two breast cancer pleural effusion samples with MRK003. Combined data (average ± SEM) from 3 assays are shown, except for patient samples, which are from single assays (hence, there are no error bars). A Student's t-test was used for statistical analysis. *p<0.05. D2: day 2; D7: day 7; LY: LY-411,575; MRK: MRK003; BC-PE1 (3), breast cancer pleural effusion #1, #3.
We next examined proliferation of the stem-like and progenitor cells treated with MRK003 or DMSO as a control using an MTT-based assay (MTS assay). Cell proliferation was significantly decreased after 48 hours (data not shown).

**Notch-1 Fc Chimera Prevents Mammosphere Formation**

The γ-secretase complex is involved in intramembranous cleavage of a number of important targets in addition to Notch, such as E-cadherin, CD44 and ERBB4 (186). Therefore, we wanted to use a different approach for inhibiting the Notch pathway. The Notch-1 Fc chimera (Notch-1 decoy) has been shown to bind Jag-1 and block Notch activation. This decoy consists of amino acids 19 – 526 of the Notch-1 extracellular domain (including the first 13 EGF repeats) fused to the Fc region of human IgG1. EGF repeats 11 and 12 of the decoy will bind to the ligand Jag-1 preventing the interaction between endogenous Jag-1 and the Notch receptor. A recombinant human IgG1 Fc protein was used as a control.

First, functional activity of the decoy in inhibiting Notch signaling was confirmed using a luciferase reporter assay similar to that already described. However, in these experiments, we co-cultured the T47D-A18 cells transfected with the Hey-1 promoter luciferase reporter construct with cells overexpressing Jag-1 to induce Notch activation. Briefly, LTK-Jag-1 (mouse L cells with a deleted thymidine kinase overexpressing Jag-1) were pre-treated with the Notch decoy (hN1-Fc) (3 µM) or hIg-Fc (3 µM) control for 15 minutes before adding them to T47D-A18 cells transfected with the luciferase reporter driven by Hey-1 described earlier. Twenty-four hours later the luciferase assay was done. There was a significant decrease in luciferase activity in the T47D-A18 cells
cultured with the decoy treated LTK-Jag-1 cells demonstrating Notch signaling was inhibited (Figure 15).

To examine the effect of the decoy on sphere formation, established mammospheres were dissociated, and re-plated in the presence of hN1-Fc (3 µM) or hlg-Fc (3 µM). Twenty-four hours later, cultures were evaluated for sphere formation. Figure 16A shows two independent experiments using T47D-C42 cells. Both the untreated and hlg-Fc treated resulted in formation of small sphere-like cell clusters whereas the Notch-1 decoy (hN1-Fc) showed primarily single cells. While all spheres were not eliminated by the Notch-1 decoy (11.7±1.3 spheres with hN1-Fc compared to 25.9 ± 4.7 with hlg-Fc p<0.05), the results showed a reduction in sphere formation (Figure 16B). The results were extended using an MTS assay to evaluate proliferation following treatment with the Notch-1 decoy, which showed a decrease in the proliferation of stem-like and progenitor cells in the presence of the Notch decoy compared to the control hlg-Fc protein (Figure 16C).
Transfection with an siNotch-1 Plasmid Inhibits Mammosphere Formation

The results were confirmed by using a short interfering RNA (siRNA) against Notch-1 to specifically inhibit Notch-1 expression. The siNotch-1 plasmid, which contains a GFP marker, was transfected into T47D-A18 cells using Lipofectamine 2000. Knockdown of Notch was confirmed by Western blotting at various time points (Figure 16: The Notch decoy protein inhibits mammosphere formation and proliferation in T47D-C42 cells. (A). Representative pictures show mammosphere formation 24 hours after treatment with 3 µM hlg-Fc, but when treated with 3 µM hN1-Fc. (B). Quantitation of mammospheres. Data shown are from two experiments ± standard deviation (SD). (C). An MTS assay revealed a significant decrease in proliferation of cells treated with the Notch decoy (3 µM) compared to the control (3 µM). Data shown are from two independent experiments ± SD.)
17A). We found that the best inhibition of Notch occurred at 96 hours. In a separate experiment, transfected cells were FACS sorted for GFP-positive and GFP-negative cells then plated as mammospheres. Cells that did not express GFP (indicating the cell did not contain siNotch-1) grew into mammospheres (Figure 17B) while GFP-positive cells which were successfully transfected with siNotch-1 remained mainly as single cells (Figure 17B). This suggests that Notch-1 was specifically involved in mammosphere proliferation.

Figure 17: Transfection of the siNotch plasmid prevents mammosphere formation in T47D-A18 cells (A) Transfection of a siNotch-1 plasmid decreases Notch expression in T47D-A18 cells as determined by Western Blotting. (B) In a separate experiment T47D-A18 cells were transfected with siNotch-1-GFP and sorted for GFP-positive and GFP-negative cells after two days. The cells were plated under mammosphere conditions and monitored for mammosphere growth. The GFP-negative cells (not successfully transfected with the plasmid) grew as mammospheres (fluorescent microscopy on the left side and brightfield microscopy on the right side). However, cells that were transfected with siNotch-1 (GFP-positive) remained as single cells. Representative pictures are shown.
MRK003 Prevents Colony Formation

Many drugs are less effective in vivo or when cells are grown as three-dimensional spheres in a matrix, which more closely mimics in vivo conditions. Therefore, we wanted to determine the effects of MRK003 on cells cultured in soft agar. Established mammospheres were prepared, dissociated, and re-plated in 0.35% soft agar containing 20 μM MRK003 or DMSO as a control. Experiments were performed with T47D-A18, MCF7, MDA-MB-231, and BT474. In every cell line tested, colonies were clearly visible in cultures treated with DMSO by day 14, while only cellular debris was present in MRK-treated cultures at the same time point (Figure 18). The number of colonies was quantified, and there was a statistically significant reduction in the number of colonies between control-treated and MRK003-treated cultures in each tested cell line (p<0.05). Colony formation assays were attempted with two samples from breast cancer pleural effusions; however, there was no colony formation in untreated or DMSO treated samples after 21 days making it impossible to draw meaningful conclusions.

Figure 18: MRK003 prevents colony formation in soft agar. Mammosphere cultures were established in soft agar in the presence of DMSO or MRK003 (10-20 μM). DMSO-treated cells formed colonies, which were not visible in MRK003-treated cultures. Representative results from day 14 are shown. Quantitation of colony formation is shown in the bar graph. The results represent combined data (average ± SEM) from 3 independent experiments. A Student’s t-test was used for statistical analysis *p<0.05.
MRK003 Induces Apoptosis in Mammospheres

As noted above, MRK003 treatment results in cultures containing debris and condensed, fragmented cells (Figure 14A-C). To determine if the cells had died via apoptosis, we performed TUNEL staining to detect the DNA strand breaks characteristic of apoptosis. T47D-A18 mammospheres were treated with MRK003 or DMSO for 24 or 48 hours and cytospin cell preparations made. Earlier time points were chosen so that any positive TUNEL staining could be detected prior to loss of the cell structure at day 7 (Figure 14C). TUNEL staining showed small mammospheres in the DMSO-treated cultures that were primarily TUNEL-negative. However, cells from MRK003-treated cultures were primarily single cells with clear evidence of TUNEL-positivity at 24 hours that increased at 48 hours (Figure 19).

Figure 19: MRK003 induces apoptosis in T47D-A18 mammospheres (A) DMSO-treated cells (B) MRK003-treated cells at 24 hours (C) MRK003-treated cells at 48 hours. Results are representative of three independent experiments. Arrows indicate examples of TUNEL positive cells.
Apoptosis in mammospheres was confirmed using Annexin V / PI staining. Annexin V identifies cells in earlier stages of apoptosis by binding to phosphatidylserine, which is normally found inside the cell but flips to the outside during early apoptosis. PI stains cellular DNA. As the cells in this protocol are not permeabilized, only injured / destroyed cells or those in late apoptosis will be PI-positive. Using mammospheres derived from T47D-A18 cells and treated for 96 hours with 20 μM MRK003, we found 64.63% ± 9.32 (p<0.02) of cells were Annexin V-positive (Figure 20). The results were confirmed with mammospheres derived from SKBR3 cells, which showed 37.64% ± 6.4 (p<0.04) of the cells were Annexin V-positive (Figure 20).
Figure 20: MRK003 induces apoptosis in T47D-A18 and SKBR3 mammospheres as determined by Annexin V / PI staining. (A) Annexin V / PI staining where the X-axis represent Annexin V and the Y-axis represent PI staining. Representative results with SKBR3 are shown. (B) Representative results with T47D-A18 cells. (C) Combined data from three independent experiments. Data is presented as the average +/- SEM. *<0.04, ** p<0.02.
The Tumor Necrosis Factor-Alpha Pathway may be Involved in MRK003-induced Apoptosis

The mechanism of apoptosis induced by MRK003 in T47D-A18 mammospheres was further elucidated. We chose to use the Human Apoptosis RT² Profiler PCR array, as it provides information on 84 apoptosis-related genes in a single experiment. RNA was extracted from T47D-A18 mammospheres treated with MRK003 (or DMSO) for 24 hours, and RT-qPCR was performed according to manufacturer’s protocol. The raw data was uploaded to a web-based analysis program from the array manufacturer that calculated the fold change in gene expression between the DMSO and MRK-treated samples.

The data show significant increases in several target genes. Of interest was the notable increase in mRNA expression of genes associated with the extrinsic apoptotic pathway in MRK-treated mammospheres compared to DMSO-treated controls. The targets and fold changes of various targets that were upregulated or downregulated are shown in Table 5. Importantly, members of the TNF-receptor family were increased, as were several caspases. A DNA damage induced gene, GADD45, was also upregulated compared to the DMSO control. Several genes were also downregulated after treatment with MRK003. Akt, which plays an important role in cell survival, and Bcl2, a pro-survival gene, were downregulated. Caspase-2 was also lower in the MRK003 treated mammospheres. Alternate names for the upregulated targets are summarized in Table 6.
Table 5: Summary of tumor necrosis factor-alpha related apoptotic genes induced following treatment of T47D-A18 mammospheres with 20 µM MRK003 for 24 hours. The table shows the average fold-increased/decrease calculated by SABiosciences web-based software from 2-3 independent experiments.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Average fold-increase</th>
</tr>
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<tbody>
<tr>
<td>Caspase 4</td>
<td>10.3-fold</td>
</tr>
<tr>
<td>Caspase 8</td>
<td>2.4-fold</td>
</tr>
<tr>
<td>Caspase 9</td>
<td>2.8-fold</td>
</tr>
<tr>
<td>Caspase 10</td>
<td>10.5-fold</td>
</tr>
<tr>
<td>GADD45A</td>
<td>32.5-fold</td>
</tr>
<tr>
<td>TNF</td>
<td>4.6-fold</td>
</tr>
<tr>
<td>TNF-R1</td>
<td>2.2-fold</td>
</tr>
<tr>
<td>DR4</td>
<td>2.4-fold</td>
</tr>
<tr>
<td>DR5</td>
<td>9.4-fold</td>
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<table>
<thead>
<tr>
<th>Gene</th>
<th>Average fold-decrease</th>
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<tbody>
<tr>
<td>Akt</td>
<td>3.95-fold</td>
</tr>
<tr>
<td>Bcl2</td>
<td>2.03-fold</td>
</tr>
<tr>
<td>Caspase 2</td>
<td>2.56-fold</td>
</tr>
</tbody>
</table>

Table 6: Alternate names for apoptotic targets upregulated in the T47D-A18 cell line after treatment with MRK003. The table provides only a few alternate names and is not comprehensive.

<table>
<thead>
<tr>
<th>Full name</th>
<th>Abbreviated Name</th>
<th>Alternate Abbreviations</th>
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<tr>
<td><strong>TNF Family</strong></td>
<td></td>
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<tr>
<td>Tumor necrosis factor receptor superfamily 1A</td>
<td>TNFRSF1A</td>
<td>TNFR-1, CD120a</td>
</tr>
<tr>
<td>Tumor necrosis factor receptor superfamily 10A</td>
<td>TNFRSF10A</td>
<td>TRAIL-R1, DR4, CD261</td>
</tr>
<tr>
<td>Tumor necrosis factor receptor superfamily 10B</td>
<td>TNFRSF10B</td>
<td>TRAIL-R2, DR5, KILLER, CD262</td>
</tr>
<tr>
<td><strong>Caspases</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caspase 4</td>
<td>CASP4</td>
<td>ICEREL-11, ICH-2</td>
</tr>
<tr>
<td>Caspase 8</td>
<td>CASP8</td>
<td>FLICE, MACH</td>
</tr>
<tr>
<td>Caspase 9</td>
<td>CASP9</td>
<td>ICE-LAP6, MCH6</td>
</tr>
<tr>
<td>Caspase 10</td>
<td>CASP10</td>
<td>FLICE-2 MCH4</td>
</tr>
<tr>
<td><strong>DNA damage response</strong></td>
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<tr>
<td>DNA-damage-inducible 45 alpha</td>
<td>GADD45</td>
<td>DDIT-1</td>
</tr>
</tbody>
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c-Jun-N-terminal Kinase and p38 Mitogen Activated Protein Kinase Inhibitors Rescue Mammospheres from the Effects of MRK003 Treatment

To complement the data gained from the PCR array experiments, we used an alternative approach to determine if signaling pathways such as JNK, p38 MAPK, ERK (extracellular signal-regulated kinase) or JAK (Janus kinase) were involved. T47D-A18 mammospheres were treated with either 10 μM MRK003 alone or in combination with one of the following inhibitors: JNK inhibitor II, SB203580 (p38-MAPK inhibitor), PD98059 (MEK inhibitor) or AG490 (JAK inhibitor). Previous data in the lab showed that the functional activity of the inhibitors at 1X and 10X demonstrated substantial blockade of the target (240).

After 24-48 hours, the number of mammospheres present in each condition was quantified. The data was analyzed at a time point when clear mammospheres were present in the DMSO control. We observed that only the JNK inhibitor 0.040 μM (1X concentration) and SB203580 (0.60 μM) (1X concentration) were able to rescue mammospheres from MRK003-induced effects (Figure 21 A, B). The other two inhibitors did not show mammosphere formation in MRK003 and inhibitor treated wells (Figure 21C and data not shown). The fact that the JNK inhibitor rescued cells from MRK003 induced effects supports data shown in Table 4, which shows an upregulation in GADD45. GADD45, has been shown to induce JNK-mediated apoptosis (222). An NF-κB inhibitor and a MEK inhibitor (PD98059) (data not shown) were also tested; neither rescued mammospheres from MRK003 induced effects. In fact, at 2X and 10X IC_{50} concentrations the NF-κB inhibitor alone blocked mammosphere formation. This would indicate that the inhibitor might be having an effect on mammosphere formation which is
consistent with published data (245). Such an effect was not seen with any of the other inhibitor.

Figure 21: c-Jun-N-terminal kinase and p38 mitogen activated protein kinase inhibitors rescue mammospheres from MRK003 effects. Mammospheres were treated with 1X or 10x IC50 values of JNK inhibitor II (A), SB203580 (B), p38 MAPK inhibitor, or (C) AG490 (JAK inhibitor) for 24 hours. The results show the average ± SEM from 4 independent experiments with the exception of AG490, which is average ± SD from two experiments. Statistical analysis was performed using a Student's t-test.
CHAPTER 4
DISCUSSION

In the last decade, strong evidence has emerged validating the existence of breast CSCs and indicates that breast cancer originates from and is maintained by CSCs (112). The CSC hypothesis has changed the way investigators are approaching development of novel therapies. CSCs are more resistant to radiation and chemotherapy, and the development of new therapies targeting these cells is essential to the eradication of breast cancer (146, 147).

One way to discover potential targets for the elimination of CSCs is to identify genes that are differentially expressed in the putative CSC. A gene signature was recently prepared for CD44⁺ CD24⁻/⁻ and mammospheres from primary tumors, and the results compared to known gene expression profiles of the different breast cancer subtypes (246). The CSC pattern most closely resembled the claudin-low phenotype. This may explain why these types of cancer are the hardest to eliminate since they contain gene expression patterns of the stem-like population (246). In another study using a genomics-proteomics approach, the gene expression patterns of CD44⁺CD24⁻/⁻ and the different molecular classifications of breast cancer were compared. It was found that metaplastic breast cancer (claudin-low) had a high ratio of CD44 to CD24. In addition, the CD44⁺ CD24⁻/⁻ genomic profile correlated with the metaplastic breast cancer and claudin-low profiles.
This indicates that these cancers express high levels of genes associated with the stem-like phenotype.

Shipitsin et al. identified a molecular signature for CD44-positive and CD24-positive cells derived from breast cancer pleural effusion samples, invasive tumors, ascites samples or normal breast tissue (247). They found that CD44-positive cells expressed high levels of stem cell markers. In addition, CD44-positive cells preferentially expressed genes associated with migration and angiogenesis including vascular endothelial growth factor, TGF-β, NF-κB, and members of the Notch pathway. TGF-β has been implicated in regulating the pluripotency of human embryonic stem cells tumorigenesis and metastasis while vascular endothelial growth factor is important in angiogenesis (248-251).

Using SP analysis, we found increased Notch-1, Notch-4, Hes-1, and Hey-1 expression in the T47D-A18 and T47D-C42 cell lines. Interestingly, T47D-C42 cells showed higher levels of Notch and target gene expression compared to T47D-A18 cells. This finding is consistent with studies by Rizzo et al. using bulk-cultured cells suggesting that ERα-negative breast cancer cells possessed higher levels of Notch signaling than ERα-positive cells (183). This is also consistent with literature which showed that elevated levels of Notch were observed in the SP of MDA-MB-453 cells (a HER2 overexpressing ERα-negative cell line) compared to non-SP cells (252). In another study, primary mammary epithelial cell cultures were prepared from mouse mammary glands, then stained with Hoescht dye and the SP vs. non-SP analyzed for various
targets. It was found that SP cells contained higher levels of the Notch target Hey-1 and higher expression of ABCG2 was reported compared to the non-SP. Other Notch targets were not explored (253). This is consistent with our data. On the other hand, Patrawala et al. tested several cell lines from different cancers, and found that cells that were ABCG2-negative (by RT-PCR) expressed higher levels of Notch-1. They also showed that for most of the cancers tested the ABCG2-negative population expressed higher levels of Oct4, a stem cell marker. However, in breast cancer, the ABCG2-positive cells (SP) contained slightly higher levels of Oct4 as compared to ABCG2-negative cells (137). Although Patrawala’s results appear contradictory to our data, their study showed Notch expression in only one breast cancer cell line (MDA-MB-435), and they did not analyze downstream targets of Notch making it impossible to draw conclusions regarding Notch activation.

Our studies examining Notch expression and activation in mammosphere-derived stem-like cells appear to further support this finding. Notch-1 and Jag-1 mRNA was increased in mammospheres derived from all tested ERα-negative breast cancer cell lines (T47D-C42, MDA-MB-231, MDA-MB-468 cells) compared to spheres derived from either ERα-positive cell line (T47D-A18, MCF7). These results are particularly interesting given that elevated Notch-1 and Jag-1 expression are known to predict outcome in breast cancer (178, 179, 254). Other Notch receptors, ligands, and targets were increased in some ERα-negative cell lines, but not others when compared to the ERα-positive lines. This was not unexpected in these culture adapted cells as Notch signaling is highly context dependent, meaning factors such as the Notch ligand and
receptor involved, expression levels of the receptor and ligand, and the cell type in which they are expressed affect the outcome of Notch activation (174, 255). Ultimately, studies examining a larger number samples, including patient samples that are not culture adapted, will be essential prior to drawing definitive conclusions.

Interestingly, members of the Notch pathway have been upregulated in several different stem-like populations. Several authors have showed elevated levels of Notch-3 in mammospheres and the CD44-positive population derived from primary samples. Dontu et al. found high levels of Notch-3 in mammospheres derived from reduction mammoplasties (128). Sansone et al. found Notch-3 was important in mammosphere proliferation (198). Finally, Shipitsin et al. showed that the CD44-positive population had slightly elevated levels of Notch-3 compared to the CD24-positive population (247). Our data shows that mammospheres derived several breast cancer cell lines (T47D-C42, T47D-A18, MDA-MB-231, SKBR3 and BT474) and a breast cancer pleural effusion (BC-PE6) had upregulation of Notch-3 mRNA compared to the bulk population. However, at most a 2.6-fold (T47D-C42) increase in Notch-3 expression was observed in mammospheres derived from this cell line. Recently, Harrison et al showed that the CD44+ CD24low ESA+ breast CSC population expressed higher levels of activated Notch-4 than other populations with these markers. These results were verified in MCF7, MDA-MB-231 and a primary sample. Interestingly, levels of activated Notch-1 were lower in the breast CSC population compared to the other populations. The authors provide interesting data, which suggests that alternate targets may be necessary depending on how the CSC is identified (256).
Our report is the first to demonstrate that a GSI can induce apoptosis in breast cancer stem-like cells. Our results with LLNle and LY-411,575 demonstrated temporary inhibition of mammosphere formation and growth arrest in the stem-like cells. Notch-1 siRNA and a Notch decoy also decreased mammosphere formation indicating that Notch inhibition was responsible for the observed affects. These findings were consistent with published results from other investigators (194, 242). In contrast, we found MRK003 induced apoptosis. This novel finding is potentially important because GSIs are currently in development by several pharmaceutical companies, and Merck Pharmaceutical has a drug with similar activity to MRK003 currently in clinical trials for breast cancer.

It is unclear why this compound induced apoptosis, while the other compounds had a temporary effect. We hypothesize that MRK003 has increased potency, solubility and stability compared to other commonly used GSIs. LY-411,575 is a highly potent GSI, particularly for in vivo experiments. In tissue culture, however, it precipitates when added to media making it difficult to determine the actual drug concentration reaching the cells. Moreover, it has a published half-life in vivo of 2 hours (257). Previous studies in our lab re-supplemented LY-411,575 every 6-8 hours (187, 189). Because each addition of LY-411,575 also adds DMSO to the media, we rapidly reached toxic levels of DMSO using this experimental design. LLNle is a commercially available tripeptide containing an aldehyde group. The aldehyde group interacts covalently with the active site of γ-secretase; therefore, once LLNle is bound to the active site, gamma secretase will not regain function. Yet, peptides like LLNle, are rapidly degraded in serum, plasma and cells, and aldehyde-containing compounds tend to have poor metabolic stability (258).
Other investigators have primarily utilized the GSI N-[N-(3,5-difluorophenacetyl-L-alanyl)-S-phenylglycine tert-butyl ester (DAPT). This peptide likely has comparable stability to LLNle as it is structurally similar. DAPT has a peak absorbance at 244nm on UV spectrometry. Liao et al. recently used DAPT and other peptidomimetics in a series of experiments and monitored changes in OD$_{244}$ as a measure of conformational integrity and chemical stability of the compounds (259). They showed that the OD$_{244}$ of DAPT decreased by 50 and 70% after 4 and 24 hours, respectively, of incubation at room temperature (259). In contrast, MRK003 has been reported to maintain activity in culture for up to 96 hours. Although we cannot rule out that an off-target affect of MRK003 is responsible for these results, it is equally plausible that MRK003 is able to more effectively inhibit Notch signaling for extended periods.

It is currently believed that combined therapeutic approaches will be necessary to effectively eliminate cancer. This approach has the advantage of targeting cancer cells based on different criteria. For example, taxanes can be used to block mitosis targeting rapidly proliferating cells along with an alkylating agent to damage DNA or bortezomib to inhibit the proteosome. Additionally, synergistic activity between different therapeutics may allow lower doses of the individual drugs to be used, which can minimize or even eliminate toxic side effects. Therefore, by combining MRK003 with a chemotherapeutic agent such as Paclitaxel, Adriamycin, Cytoxan or Taxotere, we may be able to decrease the doses and side effects of both drugs in patients. These drugs are commonly used in the clinic to treat breast cancer. Paclitaxel may be a good option because the ER status of the stem-like population is controversial. Several studies have shown that the stem-
like population (as the SP) is ERα-positive (136, 260). Other groups reported an ERα-negative SP in either mice or humans (261, 262). Additionally, mammospheres likely contain both ERα-positive and ERα-negative cells. In order to avoid any complications with regard to the ER, using chemotherapeutic agents such as those listed above, which are not dependant on the ER should prove beneficial. In fact, Dr. Jenny Chang presented recent data from her laboratory, in which breast tumors were established in mouse xenografts; once a tumor had grown to 150 mm$^3$-300 mm$^3$ treatment was given in the form of Docetaxel, MRK003 or a combination of the two. Their data showed that MRK003 was able to decrease the CD44$^+$/CD24$^{low/-}$ population, the Aldefluor-positive population, and led to a decrease in mammosphere formation in vitro. Furthermore, patients treated with GSI alone or in combination with Docetaxel resulted in their tumors containing lower levels of the stem-like population after treatment. Importantly, the tumor volume did not decrease in the mouse models, but the level of stem-like cells was decreased (263).

Our data suggest that the extrinsic pathway is involved in MRK003-induced apoptosis in mammospheres. We found TNF, TNF-R1, caspase 8 and caspase 10 mRNAs were upregulated in MRK003-treated mammospheres compared to DMSO control-treated spheres. TNF-TNF-R signaling is known to induce NF-κB and JNK signaling in addition to activating caspases 8 and 10. Moreover, studies have shown TNF-R1 is upregulated in stromal cells of breast tumors but not in normal breast tissue in a majority of samples (264). Further studies are required to determine if the MRK003-induced apoptosis can be reversed through blockade of the TNF death receptor.
pathway. It is less likely that the Fas / FasL or TRAIL death pathways are involved in apoptosis induced by the MRK GSI. Although FASL mRNA was induced, Fas expression was not altered. Similarly, TRAIL-R1 and TRAIL-R2 mRNAs were induced, but TRAIL mRNA was significantly decreased. Without expression of both the receptor and ligands, it is unlikely that these pathways play a substantial role in our system.

However, the induction of TRAIL-R1 and TRAIL-R2 remain intriguing as it may be possible to harness this death receptor pathway to augment cell killing induced by MRK003 GSI. The development of agents that engage TRAIL receptors and induce apoptosis has been an area of intensive research. TRAIL has been shown to selectively induce apoptosis in tumor cells while normal cells are relatively resistant to TRAIL-induced death (265, 266). Studies in breast cancer cell lines have shown treatment with TRAIL alone induced apoptosis in only 2 out of 16 breast cancer cell lines tested. However, by adding chemotherapeutic agents such as doxorubicin or 5-fluorouracil along with TRAIL, apoptosis was induced in all 16 breast cancer cell lines (267). The reason for this may be that certain chemotherapeutic agents may induce the expression of DR4, or DR5 making cells sensitive to TRAIL. It has also been shown that the TRAIL receptors had missense mutations in several breast cancer cell lines making them unresponsive to treatment with TRAIL (212). An alternative approach would be using TRAIL gene therapy. This therapy has been shown to be effective in cell lines that did not respond to treatment with TRAIL. An adenoviral vector, which contains the TRAIL gene driven by a human telomerase reverse transcriptase promoter is injected into the tumor in mouse xenografts. Data showed that after this therapy, there was a complete
regression of tumors in 50% of mice and tumors did not re-form for the 5 months that mice were monitored after complete regression was observed. This provides strong evidence that such a treatment could be effective (268, 269). Clinical trials have also been initiated with TRAIL receptor agonists, which showed to be effective against solid tumors, but data were preliminary. Additionally, humanized monoclonal antibodies against TRAIL-R1 and TRAIL-R2 are currently in clinical trials (270). Studies are ongoing in the Foreman laboratory to investigate combining MRK003 and TRAIL as a means to eliminate the breast CSC.

We observed that inhibition of the JNK pathway and the p38 MAPK pathway were able to reverse the effects of MRK003 on mammosphere formation indicating that these pathways may play a role in apoptosis of mammospheres. The JAK2/EGFR kinase inhibitor (AG490) and the MEK-1 inhibitor (PD98059), however, were not able to reverse the effects of MRK003. Interestingly, the NF-κB inhibitor did not reverse the effects of MRK003, but it was able to prevent mammosphere formation. This is not surprising because NF-κB is a downstream target of Notch in the non-canonical pathway. Furthermore, treatment with NF-κB inhibitors was shown to decrease proliferation of mammospheres derived from MCF7 cells, as well as, decreased the SP fraction (245).

Interestingly, the Notch and p38 MAPK pathway have been shown to interact in skeletal muscle differentiation (myogenesis). Activated Notch was shown to induce expression of mitogen activated protein kinase phosphatases-1 (MKP-1), which is a dual-specificity phosphatase that dephosphorylates and inactivates p38 MAPK preferentially in skeletal muscle (271). This is an interesting hypothesis to pursue since
both the JNK and p38 MAPK pathways are somewhat redundant in that they are involved in apoptosis and survival.

The role of MKP-1, -2 has been explored in breast cancer tumors. Malignant and non-malignant samples were taken from patients who had primary breast cancer and assayed for MAPK activity and the presence of MKP-1, and -2. Expression and activity of MKP-1, -2 were compared to a non-malignant sample from the same patient. The results showed that the tumors had a 3-fold increase in expression and activity of p38 MAPK and 2.5-fold increase in JNK-1. However, the activity of JNK was decreased in malignant samples. This discrepancy was further tested by looking at the levels of MKP-1 and MKP-2, which were found to be significantly upregulated compared to non-malignant tissue. These would then dephosphorylate JNK and lead to its lower activity thereby preventing the expression of apoptotic genes. Therefore, suppressing MKP-1 and MKP-2 may prove useful in the clinic (272).

Finally, while we have shown that apoptosis is occurring in MRK003 treated mammospheres, in part through the extrinsic pathway and that JNK, and p38 MAPK may be involved in apoptosis we have not ruled out the possibility that cells are dying by mitotic catastrophe, necrosis, or autophagy. The easiest way of ruling out mitotic catastrophe is by examining the nuclei of mammosphere cells and determining if they are micronucleated, which is characteristic of mitotic catastrophe. Further studies can be done to see if cells have arrested in G2 or M phase of the cell cycle. One way of doing this is by determining the mitotic index, which can be done by staining mammospheres with mitotic protein monoclonal-2 antibodies that identify proteins that are phosphorylated only during the M-phase (189). Some of these proteins include Hsp70,
cdc25, and DNA topoisomerase IIa. If cells express high levels of phosphorylation of these targets that would indicate they are undergoing mitotic catastrophe. To distinguish necrosis from apoptosis one can look at swelling and bursting of the cell consistent with necrosis by electron microscopy. Furthermore, since necrosis is not dependant on caspases it is unlikely that it is the major pathway occurring after MRK003 treatment, as several caspases were upregulated. Autophagy can be distinguished by using a GFP tagged-LC3 protein. This protein is found on the membranes of autophagosomes. They can be detected by fluorescence microscopy (reviewed in (273)).

Our results show an important role for Notch in the survival, proliferation and self-renewal of the breast cancer stem-like population. We have shown elevated levels of activated Notch in the putative CSC using three different techniques to enrich for the stem-like cells: SP, mammosphere formation, and Aldefluor staining. Inhibition of Notch signaling with GSIs resulted in growth arrest or, in the case of MRK003, apoptosis of the putative CSC. As GSIs are currently in clinical trials for breast cancer, our data support the notion that Notch may be a useful target for the treatment of breast cancer. One major problem in treating breast cancer is the fact that tumors are heterogeneous and generally contain both ERα-positive and ERα-negative cells, and studies have shown that approximately 20 - 30% of women with ERα-positive primary tumors develop ERα-negative metastasis (274, 275). Butler et al. simultaneously measured ER and PR status in women with node-positive breast cancer and reported 25% with ERα-positive primary tumors had an ERα-negative nodal metastasis (276). As MRK003 was able to eliminate the CSC from both ERα-positive and ERα-negative, Notch inhibition may be better able to CSCs in wide variety of breast cancer patients. Our data also show that treatment with MRK003 upregulated members of the TNFα pathway including TNF-R1 and
downregulated of several targets including Caspase-2, Bcl2 and Akt. Akt is involved in survival and can also inhibit caspase-9 by phosphorylation (277). While Bcl2 is a pro-survival protein and caspase-2 can cleave Bid to t-Bid. These data are consistent with an induction of apoptosis as some pro-survival members are downregulated. It is unclear whether caspase-2 plays an important role in the induction of apoptosis in our model since its downstream target Bid was not affected at the mRNA level, however, it may be affected at the protein level.

Our data implicate the JNK and p38 MAPK in MRK003 induced effects. Several lines of evidence support a role for JNK in MRK003 induced effects. JNK can be activated through TNF-R1 by recruiting adaptor proteins TRAF2, which can bind ASK-1 then activate MKK4/7 and JNK (228). JNK can also inhibit Bcl2 and promote the release of cytochrome c by mobilizing Bax to the mitochondria after phosphorylation of an anchor protein 14-3-3 (278). Also, we saw upregulation of the DNA-damage inducing agent GADD45 and RIPK2, which have been shown to modulate JNK activity (222, 279). Therefore, strong evidence exists to support a role for JNK in MRK-induced apoptosis.

Our research supports an important role for Notch in the survival, proliferation and self-renewal of the putative CSC population. Notch inhibition using MRK003 was effectively killed the progenitor and stem like cells derived from breast cancer cell lines and primary samples. These studies may help us in understanding the complex biology of the putative CSC so that we can design better targeted therapies to kill these cells.
Figure 22: Summary and proposed model (A) Notch ligands such as Delta or Jag are expressed at a higher level in the putative CSC population (mammospheres) vs bulk cells. Activated Notch was elevated in this population as determined by the expression of direct targets of Notch, the Hes and Hey family of genes. This can lead to survival proliferation and self-renewal of the putative CSC. Treatment with a GSI decreased downstream targets of Notch. GSIs also prevented mammosphere proliferation and self-renewal. One GSI MRK003 induced apoptosis in mammospheres. Addition of a Notch decoy or an siNotch-1 RNA also decreased proliferation and self-renewal.

(B) The apoptotic pathway induced by MRK003 includes the TNF-receptor death pathway. Several caspases including caspase 8/10 and caspase-9 were upregulated. Additionally, the JNK pathway and/or the p38 MAPK pathway may be involved in MRK003 induced effects. These pathways can be activated through the TNF-receptor pathway. Several proteins that activate JNK including RIPK2 and GADD45 were upregulated at the mRNA level. Furthermore, several proteins that are associated with survival, Akt and Bcl2, were downregulated at the mRNA level.
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VITA

The author, Peter Grudzien, was born on June 21, 1981 to Walter and Maria (Koziol) Grudzien. Peter has one sister, Aneta, who is currently a resident at Resurrection Hospital in Chicago. Peter attended Loyola University Chicago from 1999-2003 and performed research in a *Drosophila* laboratory looking at the activity of a transposable element called hobo in various *Drosophila melanogaster* fly lines. At this time Peter started a teaching assistant position for General Chemistry Lecture and Lab Courses which he continued for four years. In 2003, Peter graduated from Loyola and continued his research in the same laboratory for his Master’s thesis. His Masters project focused on the genetics of speciation between two closely related species *D. simulans* and *D. sechellia*. During his Masters program Peter was also a teaching assistant for an introductory Genetics course. In 2005, after completing his research project for his Masters thesis Peter joined the Molecular and Cellular Biochemistry Program at Loyola University Medical Center, where he entered the laboratory of Dr. Kimberly Foreman. Peter’s research focused on the Notch pathway in the putative breast cancer stem cell population. During his time in the Foreman lab Peter trained several technicians and high school summer students who participated in a research program sponsored by the American Cancer Society. Peter presented his research at two breast cancer symposia in San Antonio (2008, 2009).
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The final copies have been examined by the director of the dissertation and the signature which appears below verifies that fact that any necessary changes have been incorporated and that the dissertation is now given final approval by the committee with reference to content and form.

The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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