Inhibition of Notch Signaling Reduces the Stem-like Population of Breast Cancer Cells and Prevents Mammosphere Formation

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Abstract. *Background: Cancer stem cells (CSCs) are believed to be responsible for breast cancer formation and recurrence; therefore, therapeutic strategies targeting CSCs must be developed. One approach may be targeting signaling pathways, like Notch, that are involved in stem cell self-renewal and survival. Materials and Methods: Breast cancer stem-like cells derived from cell lines and patient samples were examined for Notch expression and activation. The effect of Notch inhibition on sphere formation, proliferation, and colony formation was determined. Results: Breast cancer stem-like cells consistently expressed elevated Notch activation compared with bulk tumor cells. Blockade of Notch signaling using pharmacologic and genomic approaches prevented sphere formation, proliferation, and/or colony formation in soft agar. Interestingly, a gamma-secretase inhibitor, MRK003, induced apoptosis in these cells. Conclusion: Our findings support a crucial role for Notch signaling in maintenance of breast cancer stem-like cells, and suggest Notch inhibition may have clinical benefits in targeting CSCs.*

Abbreviations: ALDH1: Aldehyde dehydrogenase; BC-PE: breast cancer-pleural effusion; CSC: cancer stem cell; DCIS: ductal carcinoma *in situ*; DMSO: dimethyl sulfoxide; ERα: estrogen receptor-alpha; ESA: epithelial specific antigen; FBS: fetal bovine serum; GSI: gamma-secretase inhibitor; LLNle: Z-Leucine-Leucine-Norleucine-CHO; N^{IC}: intracellular domain of Notch; RT-qPCR: quantitative reverse transcriptase polymerase chain reaction; SP: side population

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A growing body of evidence exists supporting the concept that breast cancer is initiated and maintained by a subset of self-renewing cells, called cancer stem cells (CSCs) (1). These cells have been consistently identified in established breast cancer cell lines and primary patient samples and can be identified based on functional activity (self-renewal, serial tumor propagation) and phenotypic markers ($CD44^+CD24^{-/low}ESA^+$, aldehyde dehydrogenase-1 (ALDH) activity) (2-4).

Breast CSCs appear to drive both tumorigenesis and metastases, and successful treatment of patients will require elimination of these cells. This is complicated, however, by evidence that breast CSCs are resistant to standard radiation and chemotherapy (5-7). Therefore, new therapeutic approaches need to be identified to target this population (8). One strategy is to target critical signaling pathways for self-renewal and differentiation, and several candidate pathways have been identified including Notch, Wnt, and Hedgehog (8). Notch proteins are a family of transmembrane receptors that play a fundamental role in cell fate decisions. In mammals, there are four Notch receptors (Notch 1-4) and five ligands (Jagged-1, -2, Delta like (Dll)-1, -3, -4). Notch signaling is initiated by receptor-ligand interactions between neighboring cells resulting in cleavage of the intracellular domain by the gamma-secretase complex (9). The intracellular domain (N^{IC}) then translocates to the nucleus where it activates transcription *via* CSL (also termed CBF-1 or RBP-Jκ). CSL/Notch interactions induce target gene expression including members of the Hes and Hey families of transcription factors that ultimately prevent cell differentiation (9).

Notch signaling is a known regulator of mammary gland development, where it functions in stem cell self-renewal and differentiation (10). Dontu *et al*. reported that Notch activation promoted sphere formation in cultures derived from normal breast tissue and increased the number of multilineage progenitors (10). Notch signaling is commonly activated in breast cancer, and Notch inhibition (using gamma-secretase inhibitors) can kill these cells *in vitro* and *in vivo* (11-13). Furthermore, loss of Numb expression, a negative regulator of Notch, has been described in over 50% of breast cancer cases, and overexpression of Notch-1 and Jagged-1 has been shown to correlate with a poor prognosis (14-16). The role of Notch in breast CSCs is incompletely understood. Farnie *et al*. reported mammospheres derived from ductal carcinoma *in situ* expressed more N^{IC}-1, N^{IC}-4 and the downstream target Hes-1, than those derived from normal mammary tissue, and Notch inhibition resulted in reduced mammosphere formation (17). Notch-3 has also been identified in mammospheres, and shRNA-mediated Notch-3 knockdown resulted in reduced mammosphere size suggesting a role in progenitor cell proliferation (18, 19). More recently, Harrison *et al*. reported that sphere-derived stem cells and $CD44^+CD24^{-/low}ESA^+$ cells from breast cancer cell lines and patient samples possessed elevated Notch-4, but not Notch-1, expression. They found Notch-4 inhibition was more effective in reducing tumor initiation than targeting Notch-1 suggesting breast CSCs rely preferentially on Notch-4 signaling (20).

Gamma-secretase inhibitors (GSIs) are considered pan-Notch inhibitors as they effectively block activation of all four Notch receptors. Pre-clinical studies have demonstrated that GSIs can induce growth arrest and/or cell death in various cancer cells, including breast cancer (21, 22). Consequently, these compounds have entered clinical trials, but it remains unclear how these compounds will affect CSCs. Here, we examined Notch activation and blockade in stem-like cells derived from ERα-positive (T47D-A18, MCF7), ERα–negative (T47D-C42, MDA-MB231), and Her2/neu overexpressing (BT474, SK-BR3) cell lines as well as several primary tumor specimens.

Materials and Methods

Breast cancer cell lines. T47D-A18 and MCF7 cells, ERα-positive, estrogen-responsive breast cancer cell lines, were grown under estrogen-containing conditions (RPMI-1640, 10% fetal bovine serum (FBS), 6 μg/ml insulin). T47D-C42 cells, an ERα-negative, hormone-nonresponsive breast cancer cell line, were grown under estrogen-deprived conditions (phenol red-free RPMI-1640 media, 10% charcoal-dextran stripped FBS, 6 ng/ml insulin). The T47D-A18 and T47D-C42 cell lines cells were originally subcloned from the T47D line and characterized in the laboratory of Dr. V. Craig Jordan and have been extensively used as a model of acquired resistance to endocrine therapy (23-25). Both cell lines were the kind gift of Dr. Debra Tonetti (University of Illinois at Chicago, Chicago, IL, USA) and Dr. V. Craig Jordan (Georgetown University, Washington DC). MDA-MB231 breast cancer cells, an ERα-negative, basal-type cell line, were grown in IMEM containing 5% FBS. MDA-MB468 cells, another ERα-negative, basal-type line, were grown in DMEM-Ham's F12 (50:50) and 10% FBS. BT474 and SKBR3 breast cancer cells, which both overexpress Her2/neu, were grown in IMEM containing 10% FBS. All cell culture media was also supplemented with 1% nonessential amino acids, 2 mmol/l L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. MDA-MB231, MCF7, SKBR3 and BT474 cells were the kind gift of Dr. Clodia Osipo (Loyola University Chicago, Chicago, IL, USA) and MDA-MB468 cells were purchased from ATCC (Manassas, VA). To ensure the integrity of our cell lines, we continuously monitor them for changes in cellular morphology and growth characteristics as recommended by the ATCC (Cell Line Verification Test Recommendations technical bulletin). Mycoplasma testing is performed on a biweekly basis using the MycoAlert Mycoplasma Detection kit (Lonza, Rockland, ME) following the manufacturer's instructions.

Primary breast cancer cells. Pleural effusions were collected from patients undergoing thoracentesis for metastatic breast cancer with informed consent. Effusions were diluted 1:1 in sterile phosphate-buffered saline (PBS), the cells pelleted, and resuspended in sterile PBS. The cells were layered on a Ficoll-Paque (GE Healthcare Life Sciences, Piscataway, NJ, USA) density gradient and collected from the interface following a 30-minute centrifugation at 500 ×*g* at room temperature. The cells were washed in sterile PBS prior resuspension in mammosphere media or use in assays. All of the patients were heavily pretreated for their malignancies and were suffering from drug-resistant disease. Four out of the six specimens were successfully used to form mammospheres and were of the following breast cancer subtypes: two ERα-positive, one triple negative, and one Her2/neu overexpressing.

Solid tumor samples were collected from three ERα-positive patients. The tissue was minced and incubated overnight with collagenase/hyaluronidase (Stem Cell Technologies) at 37˚C with agitation. The digested sample was centrifuged at 80 ×*g* for 2 minutes, and the pellet treated for 2 minutes with trypsin. The samples were then centrifuged at 350 ×*g* for 5 minutes and the pellet treated with dispase and DNASe I prior to passage through a 40 μm filter. The cells were washed, counted and placed into mammosphere culture. Mammospheres formed from two of the three samples.

Sphere formation. Primary mammospheres were generated from bulk cultured cells by seeding ultra-low attachment plates (Corning, Lowell, MA, USA) with approximately 20,000 cells/ml in mammosphere media [mammary epithelial basal media (Lonza), B27 (Invitrogen), 20 ng/ml EGF (BD Biosciences), 20 ng/ml bFGF (BD Biosciences), 4 μg/ml heparin (Sigma-Aldrich)] (26). To passage the cells, spheres were collected by gentle centrifugation, incubated for 3-5 minutes in 0.125% trypsin, and the 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 prior to plating at 1,000 cells/cm^2 in mammosphere media on ultra-low attachment plates (26). All studies were performed with either secondary or tertiary mammospheres, based on published data demonstrating primary mammospheres contain a number of contaminating, differentiated cells until passaged to secondary mammospheres, and the number of stem-like cells declines appreciably after four or five passages (26, 27).

In experiments examining the effect of GSIs on sphere formation, established mammospheres were treated for 24 hours with GSIs or DMSO as a control. The spheres were then dissociated and new mammosphere cultures established in the presence or absence of fresh GSI or DMSO. Sphere formation was evaluated every 48 hours for up to 12 days and quantitated by counting spherical cell clusters (>60 μm, in general) in 10 random, high-powered fields per experiment (17). Additional drug was added as required based on the stability of the GSI (see Results). In studies using the recombinant human Notch1-Fc protein (R&D Systems, Minneapolis, MN, USA), mammosphere were dissociated and the Notch decoy or control protein added when the new mammosphere cultures were initiated. Here, sphere formation could only be evaluated at a 24 hour time point as we were unable to re-treat the cells with additional recombinant protein, which would have been necessary to ensure continued Notch inhibition.

ALDH activity. The Aldefluor assay was used to identify and isolate stem-like cells from primary breast cancer pleural effusion samples as previously described (28). The assay was performed according to the manufacturer's instructions, and the ALDH–positive population identified by comparing the flow profiles of tumor cells incubated with and without an ALDH inhibitor (diethylamino-benzaldehyde).

Notch inhibitors. Three structurally distinct GSIs were used in our studies: Z-Leu-Leu-Nle-CHO (LLNle; EMD Biosciences, San Diego, CA, USA), LY-411,575, or MRK003. All three agents were suspended in DMSO and stored at –20˚C. A specific Notch decoy protein, recombinant human Notch-1 Fc chimera (R&D Systems) was also utilized. This protein 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. A recombinant human IgG1 Fc protein (R&D Systems) was used as a control. Both recombinant proteins were resuspended in culture media and used at a final concentration of 3 μM.

To specifically inhibit Notch-1 expression, a plasmid-based construct encoding for Notch-1 siRNA (pLVTHM-GFP-shNotch1) or an empty vector control (pLVTHM-GFP) was used. Bulk T47D-A18 cells were transfected using Lipofectamine 2000 following the manufacturer's instructions. At 48 hours after transfection, the GFP-positive cells were collected using FACS, and placed in culture under mammosphere conditions. After 48 hours (96 hours after transfection), the cells were examined for sphere formation and the results documented.

Immunostaining. Cytospin cell preparations were fixed in 50:50 methanol:acetone or 4% formalin and immunostained using a highly sensitive avidin-biotin immunoperoxidase technique (Vectastain ABC ki; Vector Laboratories, Burlingame, CA, USA) (21). Primary antibodies were purchased from Leica Microsystems (Bannockburn, IL, USA) and consisted of cytokeratin (CK) 5, epithelial-specific antigen (ESA), CK18, and CK14. The control sample represents an irrelevant isotype matched antibody. DAB (Vector Laboratories) was used as the substrate, and the cells counterstained with hematoxylin prior to mounting.

Quantitative reverse transcriptase polymerase chain reaction (RT-qPCR). Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA). Complementary DNA was synthesized by reverse transcription using oligodT and random hexamers as primers (Taqman Reverse Transcription kit, Applied Biosystems, Foster City, CA, USA), and qPCR performed using an Applied Biosystems 7300 sequence detection system with Quantitect SYBR Green PCR reagents and Quantitect Validated Primer Sets (Qiagen). 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 uracil *N*-glycosylase to destroy previous amplified product. Negative control samples containing yeast RNA (250 ng/ml) instead of cDNA were used with each primer set to monitor for possible PCR contamination. Additionally, a dissociation curve was run for each experiment, and consistently showed a single peak indicating a lack of non-specific amplification. 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 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) (29).

Luciferase assays. The Hey-1 luciferase reporter construct was the kind 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 upstream of the luciferase gene in the promoterless vector, pLuc. T47D-A18 and T47D-C42 cells were transfected with the Hey1-luc construct using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. Transfected cells were allowed to recover overnight and were then treated with Notch inhibitors. Luciferase activity was measured after 24 hours using the Steady-Glo Luciferase Assay (Promega, Madison, WI, USA) and a Turner Biosystems Veritas microplate luminometer. Due to problems with promoter crosstalk, we were unable to use a dual luciferase reporter system to measure both Notch-responsive luciferase activity and transfection efficiency (21). Therefore, control cells were transfected with a GFP expression plasmid in every experiment and used to calculate transfection efficiency, and the average value used to normalize the data as described (21).

Western blot. Whole cell extracts were prepared by lysing cells in RIPA buffer containing a mixture of protease inhibitors (Mini-Complete, Roche Molecular Biochemicals) followed by brief sonication, and centrifugation to remove insoluble cellular debris. Protein concentration was determined using a Bradford Assay (Bio-Rad Laboratories, Hercules, CA, USA). 50 μg of protein was loaded onto a NuPAGE 7% Tris-Acetate Gel (Invitrogen), transferred to an Immobilon-P (polyvinylidene difluoride) membrane, and blocked with 5% powdered milk in TBST (50 mM Tris, pH 7.5, 150 mM NaCl, 0.01% Tween-20). The membrane was then incubated with 2.5 μg/ml anti-Hes-1 antibody (ab5265, Abcam) diluted into 5% powdered milk in TBST, washed extensively, and incubated with HRP-conjugated anti-mouse IgG secondary antibody (Amersham Biosciences, Piscataway, NJ, USA). Proteins were visualized with Pierce SuperSignal West Dura substrate and a Fujifilm LAS-3000 luminescent image analyzer). Following documentation of the results, β-tubulin expression (Clone B512; Sigma-Aldrich) was evaluated as a control for protein loading. Due to differences in the size of Hes-1 and tubulin, both proteins could be detected without stripping the blot. Differences in protein expression were determined by densitometry analysis using ImageJ Software (National Institutes of Health, USA).

Colony formation assays. Mammosphere media containing 0.5% Nobel Agar (500 μl) was placed in wells of a 24-well tissue culture plate and allowed to solidify. Established mammospheres were treated for 24 hours in culture with MRK003 GSI or DMSO as already described. The mammospheres were then collected, disassociated and resuspended at 5000 cells/ml in mammosphere media containing 0.3% Nobel Agar and supplemented with MRK003 GSI or DMSO. 500 μl of the cell-agar suspension was layered on top of the 0.5% agar and allowed to solidify. Plates were incubated at 37˚C in 5% CO_2 and were examined every other day for up to 28 days. Every five days, 500 μl of fresh media with GSI or DMSO was added on top of the agar. The plates were examined every other day and photographed at regular intervals for up to 28 days. The results were quantitated by counting colonies in 10 random high powered fields.

Proliferation assay. Mammosphere cultures were established in Corning ultra-low attachment 96 well plates in the presence or absence of Notch inhibitors (GSIs or Notch1-Fc decoy protein) or appropriate control agents. After 24-48 hours, the CellTiter96 A_{queous} One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) was used to quantitate proliferation by adding 20 μl of reagent directly to wells. 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 CellTiter96 A_{queous} One Solution without cells.

Apoptosis assays. TUNEL staining was performed using the ApopTag Peroxidase *In Situ* Apoptosis Detection kit (Millipore). Mammospheres derived from T47D-A18 and MCF7 cells treated with MRK003 or DMSO for 24 or 48 hours were collected and used for cytospin cell preparations. The cells were fixed in 10% neutral buffered formalin and stained for DNA strand breaks associated with apoptosis following the manufacturer's instructions. DAB (3,3'-diaminobenzidine) was used as the substrate and the cells were counterstained with methyl green (Vector Laboratories, Burlingame, CA, USA).

The ApoScreen Annexin V Apoptosis kit (Beckman Coulter, Brea, CA, USA) was also used to detect apoptotic cells. Mammospheres from T47D-A18, MDA-MB231, and SKBR3 cell lines were treated with MRK or DMSO for 48-96 hours, and then collected and dissociated as described above. Staining of the single cell suspensions with Annexin V-FITC and propidium iodide was performed as recommended by the manufacturer.

Statistical analysis. A Student's *t*-test was used for statistical analysis. A *p*-value of <0.05 was considered significant.

Results

Identification and enrichment of stem-like cells. Generation of mammospheres from single cells cultured under non-adherent conditions in selective media is a widely used approach for enrichment of stem-like cells from cell lines and primary tumor samples. This method is particularly useful as it can also assess self-renewal of stem-like cells and progenitor cell proliferation (17). First, we established mammosphere cultures with T47D-A18 cells using published protocols. Cell clusters could be identified within 24 hours, and after 48 hours, spheres ≥60 μm were evident as determined with an eyepiece reticle and stage micrometer. To characterize the mammosphere cells, cytospin cell preparations were immunostained for markers of differentiated mammary luminal epithelial cells (cytokeratin (CK) 18, ESA), differentiated myoepithelial cells (CK14, CK10), and progenitor cells (CK5, CK10, CK14, ESA) (17, 26). Consistent with published results, sphere cells where positive for CK5 (~25% positive), CK18 (>95% positive), ESA (>95% positive) and CK14 (~25% positive), and a small portion of the cells expressed faint CK10 indicating a heterogenous cell population (Figure 1A, data not shown) (17, 26). Using the same technique, we cultured spheres from six additional cell lines (T47D-C42, MCF7, MDA-MB231, MDA-MB468, BT474, SKBR3) and six of nine primary samples from patients with either metastatic breast cancer pleural effusions (four of six samples) or solid breast tumors (two of three samples). The spheres derived from each cell type were similar to the T47D-A18, with the exception that some primary tumor samples required more time (about 72 hours) to develop spheres ≥60 μm compared with cultured cell lines. Immunostaining on cytospins of spheres derived from select cell lines and patient samples showed similar results to the T47D-A18 cells (Figure 1B, data not shown), although we were only able to evaluate CK5 expression with the primary samples due to a limiting number of cells. Figure 1B shows CK5 expression (~15-25% positive) in sphere cells derived from ERα-positive solid tumor samples.

To confirm mammospheres are enriched for stem and early progenitor cells, we examined expression of the stem cell marker, Nanog, in mammospheres and bulk cultured cells. Figure 1C shows relative mRNA expression of Nanog in the mammosphere-derived cells prepared from a series of breast cancer cell lines and a primary patient sample, BC-PE6. Nanog mRNA expression in the bulk tumor cells was set to 1.0 and the relative mRNA expression in the mammospheres graphed. Significantly elevated levels of Nanog were identified in a majority of the mammospheres (Figure 1C). Only MDA-MB231 and MDA-MB468 stem-like cells did not show increased Nanog. While somewhat surprising, the stem-like and progenitor cells from these two ERα-negative, basal type tumor lines may rely on other stem cell-associated transcription factors for self-renewal.

As an alternative, ALDH activity was used to identify and sort for the putative CSC in primary breast cancer pleural effusions. We found an average of 3.1±0.4% ALDH-positive cells in our specimens. Nanog mRNA expression was significantly increased in the ALDH-positive cells with a 2.7±0.1-fold increase in expression compared to ALDH-negative cells (Figure 1C, $p<0.05$).

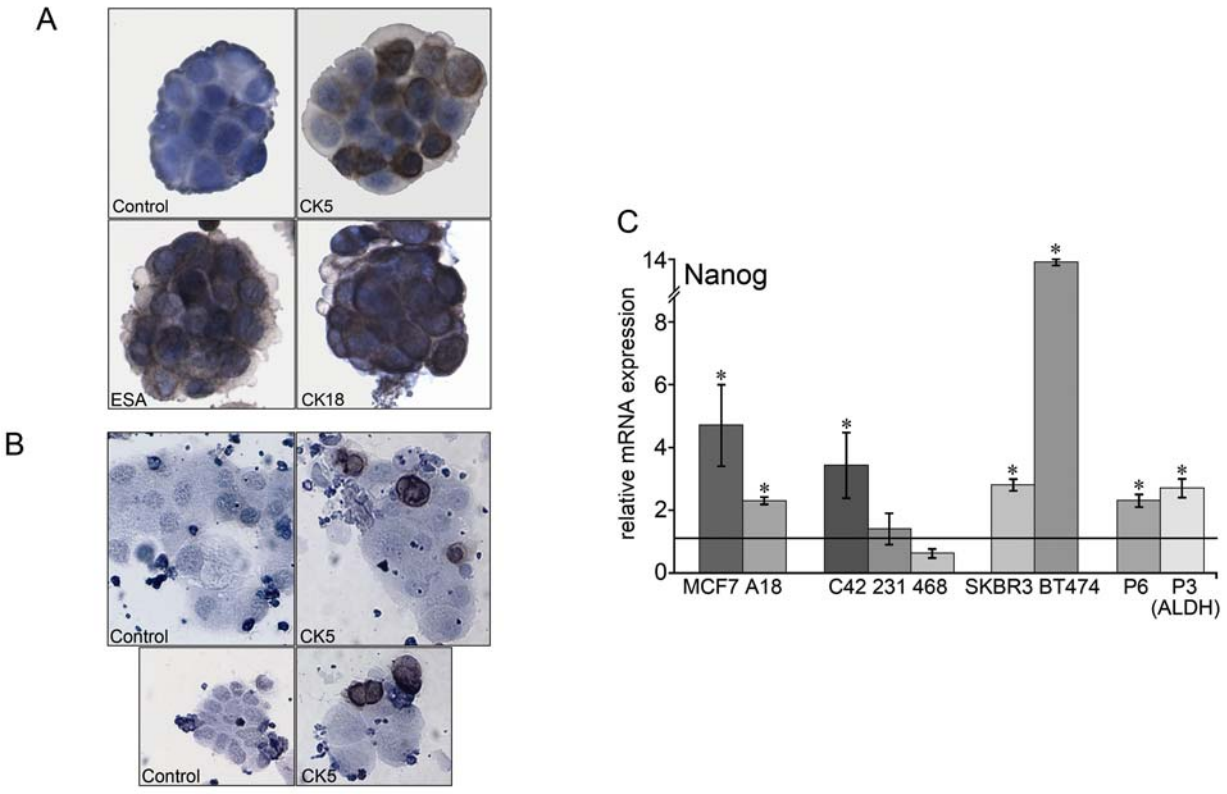

Figure 1. *Characterization of mammospheres. A: Immunostaining of mammospheres derived from T47D-A18 cells were positive for CK5, ESA, and CK18, consistent with published findings (17, 26). Results are representative of 3 independent experiments. B: Spheres derived from solid tumor specimens were also positive for CK5. The debris present in the samples is likely residual material from the collagenase/hyaluronidase digestion of the tumor, and the sphere morphology is distorted as the samples are cytospin cell preparations. Representative results are shown. C: Mammospheres derived from a series of breast cancer cell lines and a primary specimen preferentially express Nanog, a stem cell marker, compared to bulk tumor cells. Expression of Nanog mRNA in bulk cells was set to 1.0 and is indicated by the horizontal line. Relative mRNA expression in mammospheres was calculated by the comparative method and was plotted. The last sample, P3 (ALDH), represents relative mRNA expression of Nanog in ALDH-positive cells (compared to ALDH-negative) from BC-PE3, a primary patient sample. The results generated from cell lines represent combined data (average±standard error of the mean (SEM)) from 3 independent experiments where each qPCR assay was performed in triplicate. Statistical analysis was performed using a Student's t-test. Samples in which mRNA expression was significantly increased ($p<0.05$) in the stem-like population compared to bulk tumor cells are marked with an asterisk. For the patient samples, data from a single experiment is shown, where the error bars represent the standard deviation between triplicate samples. CK5: Cytokeratin 5, CK18: cytokeratin 18, ESA: epithelial-specific antigen, A18: T47D-A18, C42: T47D-C42, 231: MDA-MB231, 468: MDA-MB468, P6: BC-PE6 (breast cancer pleural effusion #6), P3 (ALDH): BC-PE3.*

Notch signaling is increased in breast cancer stem-like cells. To evaluate Notch signaling, we compared expression of a panel of Notch-associated genes in stem-like cells derived from mammospheres with bulk tumor cells using RT-qPCR. Notch receptor and ligand mRNA expression was evaluated; however, their expression does not necessarily indicate pathway activation (9). Therefore, we also examined a series of immediate downstream targets of Notch (Hes-1, Hes-5, Hey-1, Hey-2, HeyL, Deltex), which are commonly used as surrogate markers for pathway activation. A panel of breast cancer cell lines was analyzed representing different breast cancer phenotypes [ERα-positive (T47D-A18, MCF7), ERα-negative (MDA-MB231, MDA-MB468, T47D-C42), Her2/neu overexpressing (SKBR3, BT474)] and a primary tumor sample from an ERα-positive breast cancer pleural effusion (BC-PE6).

Figure 2 (panels A-D) shows relative mRNA expression of Notch-associated genes in mammospheres prepared from representative cell lines of different breast cancer phenotypes and a primary tumor sample. The results demonstrate that mammosphere-derived stem-like cells possess elevated Notch activation and preferentially express Notch receptors and ligands compared to bulk tumor cells. At least one receptor and one ligand were significantly upregulated in mammospheres compared to bulk cells (Figure 2 A-D); however, each cell line expressed a unique

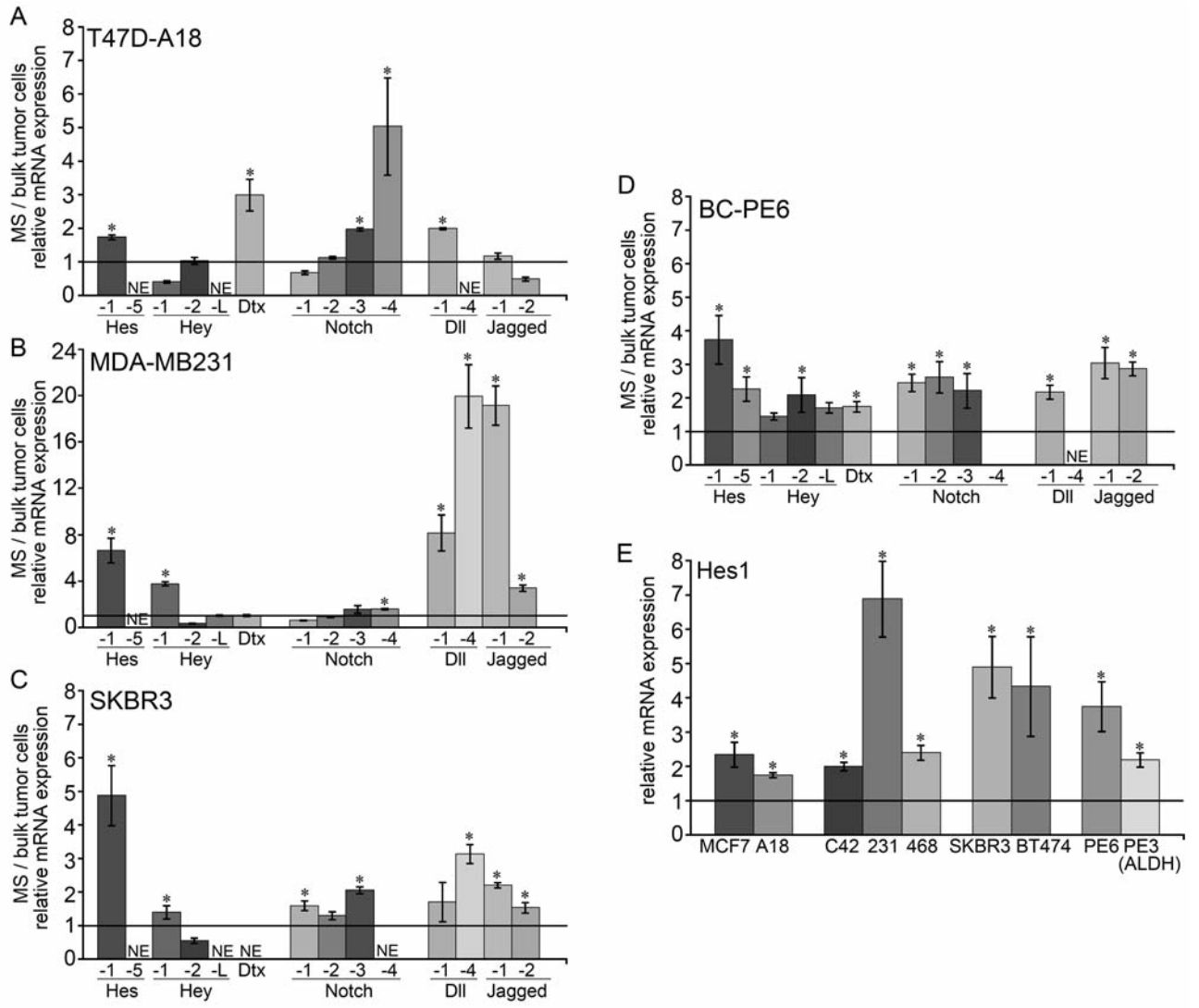

Figure 2. *Expression of Notch-associated genes in the putative breast CSCs. A-D: Mammosphere-derived stem-like cells express Notch receptors, ligands and several downstream targets of Notch signaling compared with bulk tumor cells. The results from 3 breast cancer cell lines and a primary patient specimen are shown. E: Hes-1 mRNA expression was significantly elevated in all tested samples. The results from the cell lines represent combined data (average±SEM) from 3 independent experiments each run in triplicate. Statistical analysis was performed using a Student's t-test (*p<0.05). For the patient sample, data from a single experiment is shown, where the error bars represent the standard deviation between triplicate samples. Dtx: Deltex, MS: mammosphere; NE: not expressed, A18: T47D-A18, C42: T47D-C42, 231: MDA-MB231, 468: MDA-MB468, P6: BC-PE6 (breast cancer pleural effusion #6), P3 (ALDH): BC-PE3.*

combination of receptor/ligand mRNAs. The results were similar in the other tested cell lines (MCF7, T47D-C42, MDA-MB468, BT474) (data not shown). Similarly, mammospheres expressed elevated levels of Notch target genes indicating preferential Notch activation compared to bulk tumor cells. With the notable exception of Hes-1, the combination of downstream targets expressed in the stem-like population was different for each cell line (Figure 2 A-D). Interestingly, Hes-1 mRNA was consistently and significantly elevated in mammosphere derived stem-like cells (Figure 2 E, $p<0.05$) in all tested samples. This may indicate Hes-1 is a particularly important Notch target gene in the maintenance of 'stemness'.

To compare these results to stem-like cells enriched using an alternative method, Hes-1 expression was examined in ALDH–positive and ALDH-negative cells from an ERα-positive primary specimen (BC-PE3). The ALDH–positive fraction also possessed significantly elevated levels of Hes-1 (2.1±0.1-fold increase, $p<0.05$) compared to the ALDH–negative population.

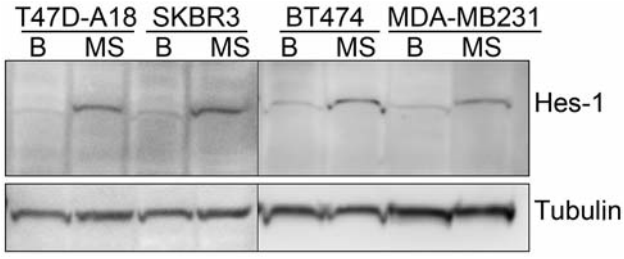

Figure 3. *Western blot analysis for Hes-1 expression. Total cellular proteins isolated from mammospheres and bulk tumor cells from representative breast cancer cell lines were analyzed. Tubulin is shown as a protein loading control. The data are representative of 3 independent experiments. B: Bulk tumor cells; MS mammospheres.*

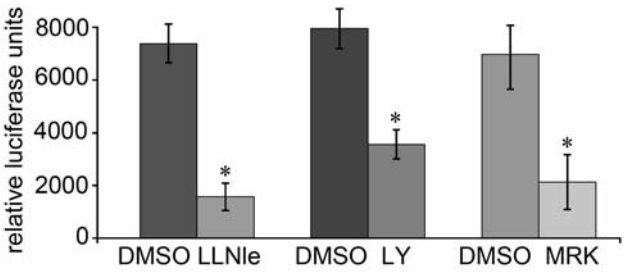

Figure 4. *Luciferase activity in T47D-C42 cells transfected with a Notch-responsive luciferase reporter. There was a significant reduction in luciferase activity in cells treated with 0.5 μM LLNLe, 25 μM LY-411,575, and 10 μM MRK003 (average reduction: 6.7-fold, 3-fold, and 5.6-fold, respectively) compared to DMSO-treated cells. Combined data (average±SEM) from 2-3 independent experiments performed in triplicate is shown. Luciferase values were normalized for transfection efficiency. Statistical analysis was performed using a Student's t-test (*p<0.05). Abbreviations: LY: LY-411,575, MRK: MRK003.*

Finally, we confirmed our findings by Western blot using representative breast cancer cell lines and comparing Hes-1 expression in mammospheres and bulk tumor cells (Figure 3). Using densitometry, we calculated the following increases in Hes-1 expression in mammospheres: T47D-A18: 3.3±0.3-fold increase ($p<0.05$); SKBR3: 3.2±0.6-fold increase ($p<0.05$); BT474: 1.7±0.1-fold increase ($p<0.05$); MDA-MB-231: 2.2±0.2-fold increase ($p<0.05$). Taken together, our results indicate breast cancer stem-like cells differentially express and activate Notch compared to bulk tumor cells.

Gamma-secretase inhibitors prevent sphere formation. Next, we examined the effect of three different GSIs (LLNle, LY-411,575, MRK003) on sphere formation. LLNle is a potent inhibitor of gamma-secretase; however, the aldehyde group of this tripeptide is able to covalently inhibit other proteases when used at >5-10 μM concentrations in breast cancer cell lines (LM, unpublished data). Even though our studies were performed with 0.5 μM LLNle, well below the concentration needed to inhibit proteases, we utilized two structurally distinct, non-aldehyde-containing GSIs, LY-411,575 and MRK003, to confirm the results (30, 31). Initial studies confirmed the GSIs significantly reduced Notch activation under our experimental conditions. T47D-C42 cells were transfected with a Notch-dependent luciferase reporter construct where luciferase expression is driven by the Hey-1 promoter. The cells were treated with LLNle (0.5 μM), MRK003 (10-20 μM), LY-411, 575 (25-50 μM) or equivalent concentrations of DMSO as a control, and luciferase activity measured 24 hours after treatment. Each GSI significantly blocked luciferase activity indicating successful Notch inhibition with an average reduction of 6.7-fold for 0.5 μM LLNle, 3-fold for 25 μM LY-411,575, and 5.6-fold for 10 μM MRK003 (Figure 4).

Next, we tested the effect of the GSIs on sphere formation beginning with T47D-A18 and T47D-C42 cells. Established spheres were treated with GSIs or DMSO, dissociated, and new mammosphere cultures initiated in the presence of fresh drug as described in the Materials and Methods. Forty-eight hours after the new cultures were initiated, small spheres were apparent in untreated and DMSO-treated samples (Figure 5A, 5E). In contrast, LLNle, LY-411,575, and MRK003-treated cultures contained primarily single cells with occasional doublets, triplets, or small spheres (Figure 5A, 5E and data not shown). Quantitation revealed significant inhibition of sphere formation with LLNle and MRK003 in comparison to DMSO-treated controls with both the cell lines (Figure 5A, $p<0.05$). LY-411,575 also inhibited sphere formation, but the reduction was more limited and not statistically significant in T47D-A18 cells (Figure 5A). This was not unexpected given the short, 2-hour half-life of this compound (32). Moreover, we have previously reported solubility issues with LY-411,575 that may adversely affect the concentration of drug actually delivered to the cells *in vitro* (21). As a control, we tested the effect of several other inhibitors targeting key signal transduction pathways on sphere formation. We have previously shown functional activity of these inhibitors and used each inhibitor at the published IC_{50} concentration and 10 times the IC_{50} (33). No difference was detected in mammosphere formation between DMSO-treated cultures and those treated with a MEK1 inhibitor (PD98059), PI3K inhibitor (LY294001) or a JAK2 inhibitor (AG490) (Figure 5B).

Blockade of sphere formation was temporary in samples treated with LLNle or LY-411,575, and spheres reformed by day 5 if additional drug was not added (data not shown). In contrast, T47D-A18 and T47D-C42 cells treated with MRK003 showed no evidence of sphere formation, and after 7 days, the treated cultures contained only debris and granular, condensed 'cells' with ragged edges that appeared

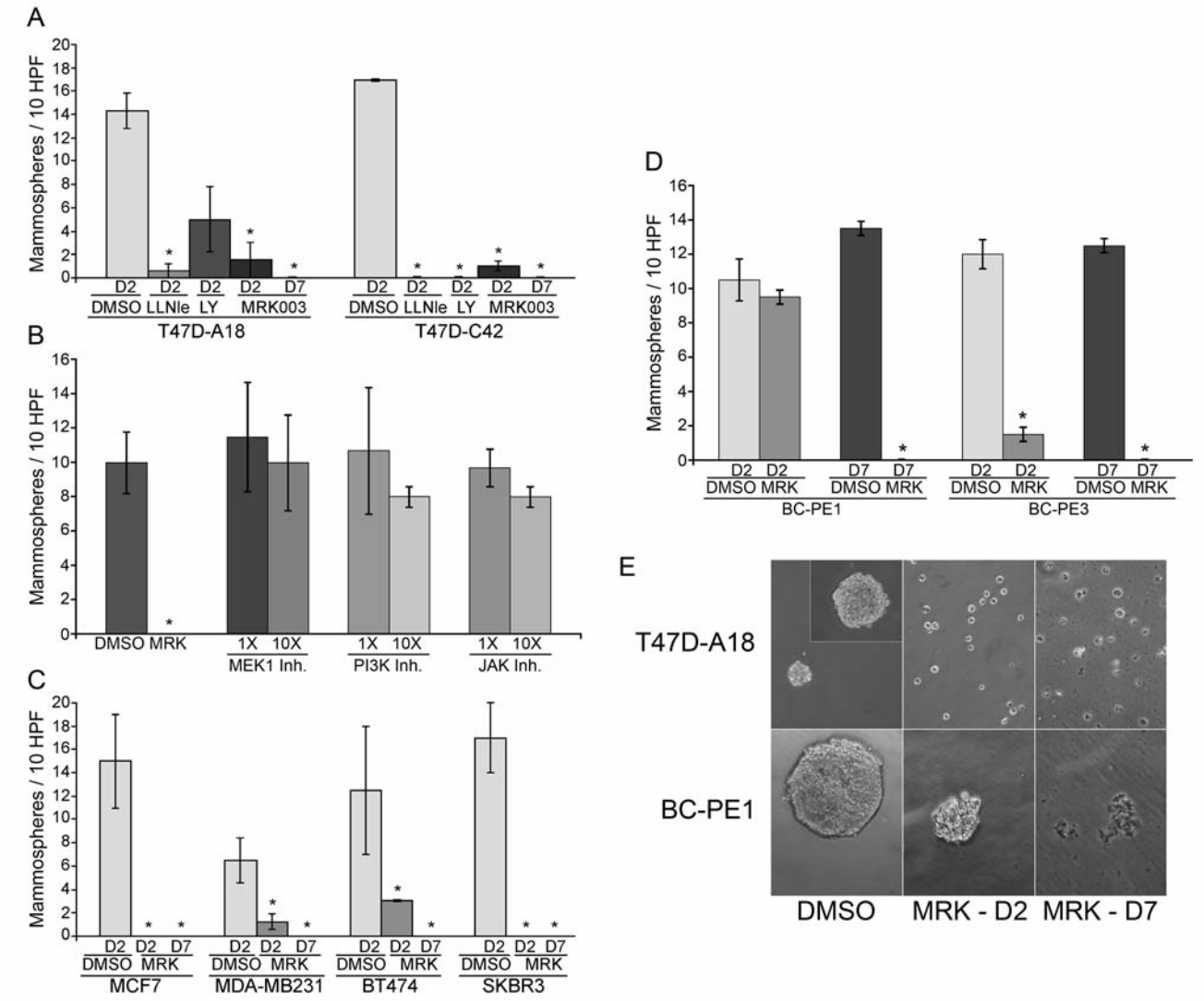

Figure 5. *Gamma secretase inhibitors prevent mammosphere formation. A-D; Mammosphere formation was quantitated in cultures treated with 0.5 μM LLNle, 25-50 μM LY-411, 575 or 10-20 μM MRK003 or DMSO as a control for 2 or 7 days. As an additional control, sphere formation was quantitated in cultures treated with 2-20 μM MEK1 inhibitor (PD98059), 1.4-14 μM PI3K inhibitor (LY294001) or 0.1-1 μM JAK2 inhibitor (AG490). Combined data (average±SEM) from 3 independent experiments is shown for cell lines. For patient samples, data is from a single experiment where the error bars represent standard deviation between triplicate samples. Statistical analysis was performed using a Student's t-test (*p<0.05). E: Representative photographs illustrating the appearance of cells treated with DMSO or MRK003. For T47D-A18 cells, the first panel represents sphere formation after two days of DMSO treatment while the inset shows a larger sphere from day 7. For BC-PE3, a mammosphere from day 7 of DMSO treatment is shown. Objective magnification: ×10. MRK: MRK003, D2: day 2, D7: day 7.*

dead (Figure 5E and data not shown). No evidence of sphere formation or cell proliferation was evident in these cultures after 14 days despite the addition of fresh growth media (data not shown).

To confirm and extend these results, cell proliferation was assessed using an MTT-based assay. As expected, 48-hour treatment of mammosphere cultures with MRK003 significantly reduced cell proliferation. In both T47D-A18 and T47D-C42 cells, DMSO-treated samples had an average absorbance of 1.33±0.1, which was reduced to 0.4±0.05 (T47D-A18, $p<0.05$) and 0.07±0.03 (T47D-C42, $p<0.05$) following MRK003 treatment.

Since MRK003 appeared to be the most promising GSI under our experimental conditions, additional studies were performed with this compound in an extend series of cell lines (MCF7, MDA-MB231, BT474, and SKBR3) and several primary breast cancer specimens. MRK003 abolished sphere formation resulting in cell death after 7-10 days in all

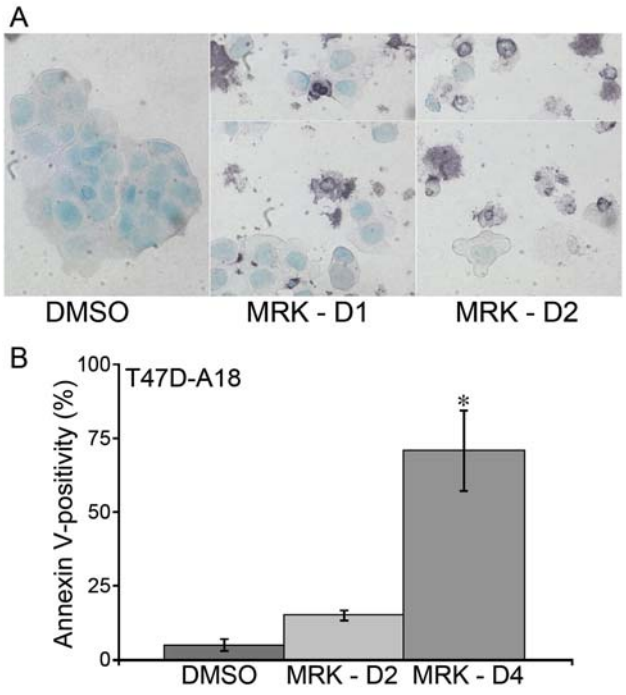

Figure 6. *MRK003 induces apoptosis in mammosphere-derived stem-like cells. A: TUNEL staining of T47D-A18 cells treated with DMSO or 20 μM MRK003. Results are representative of 3 independent experiments. Objective magnification: ×10 B: Annexin V-propidium iodide staining of T47D-A18 cells treated with DMSO or 20 μM MRK003. Combined data from 2-3 independent experiments is shown. Statistical analysis was performed using a Student's t-test. *p<0.05. D1: day 1, D2: day 2; D4: day 4, MRK: MRK003.*

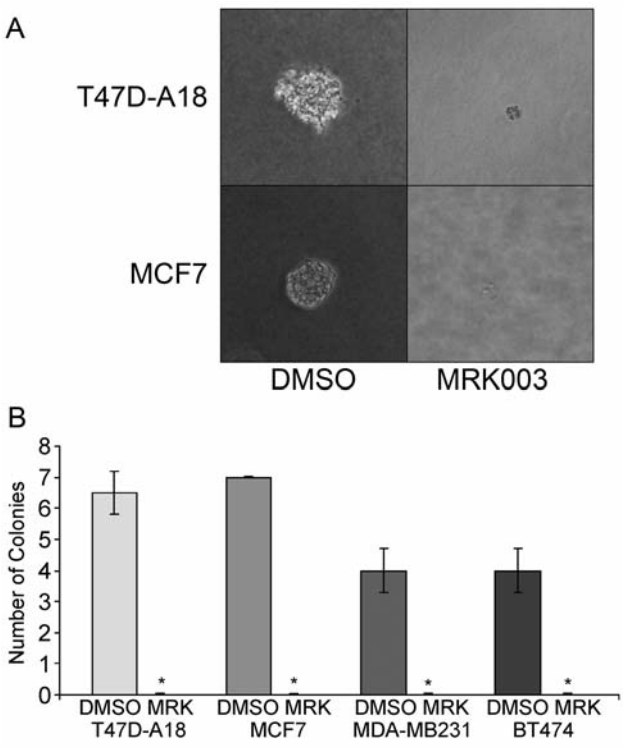

Figure 7. *Gamma secretase inhibitors prevent colony formation in soft agar. A: Representative photographs illustrating the appearance of cells treated with DMSO or MRK003 (10-20 μM) for 14 days. Objective magnification: ×10. B: Quantitation of results to colony formation assays. The results represent combined data (average±SEM) from 3 independent experiments. Statistical analysis was performed using a Student's t-test (*p<0.05).*

tested cell lines, four of six breast cancer pleural effusions and one of two solid tumor samples (Figure 5C-5E and data not shown). In all three of the primary specimens (two pleural effusion and one solid tumor) that were negative, spheres did not form in untreated control samples making the data impossible to interpret.

Interestingly, in BC-PE1 (Figure 5E), small spheres were visualized after 2 days of MRK003 treatment, where samples from cell lines and other patient samples showed only single cells. However, this did not affect the outcome. The spheres were no longer apparent at day 4, and there appeared to be complete cell death by day 8. To determine if MRK003 could eliminate the stem-like and progenitor cells in established spheres, we treated secondary mammospheres with 10-20 μM MRK003 without dissociation of the cell clusters. At day 4, cells treated with 20 μM MRK003 remained in clusters, but the cells were visibly 'stressed' and contained numerous vacuoles and appeared more granular. At day 6, cell clusters were no longer apparent, and the cultures contained only single cells and debris. No viable cells remained after 8 days (data not shown). The results were similar with 10 μM MRK003, with the exception that it took an additional two days for the cells to be eliminated (data not shown).

To determine if the cells were dying *via* apoptosis, mammosphere cultures derived from T47D-A18 cells treated with DMSO or MRK003 for 24 and 48 hours were immunostained for the DNA strand breaks typical of apoptosis (TUNEL staining). The results showed only 2.5±1.6% of cells in the DMSO control samples were TUNEL-positive compared with 28.6±1.6% ($p<0.05$) in MRK003-treated cells (Figure 6A). Similar results were found using MCF7 cells (data not shown). The results were confirmed using annexin V-propidium iodide staining where MRK003 treatment for 48 hours resulted in modest Annexin V positivity (11±1.7%) that increased dramatically by 96 hours (70.7±13.6%, Figure 6B).

In the majority of cell lines, 10 μM MRK003 was sufficient to rapidly abolish sphere formation and induce complete death of the culture in 7 days. However, some cultures, including some primary specimens, appeared more resistant and took additional time (up to 10 days) for

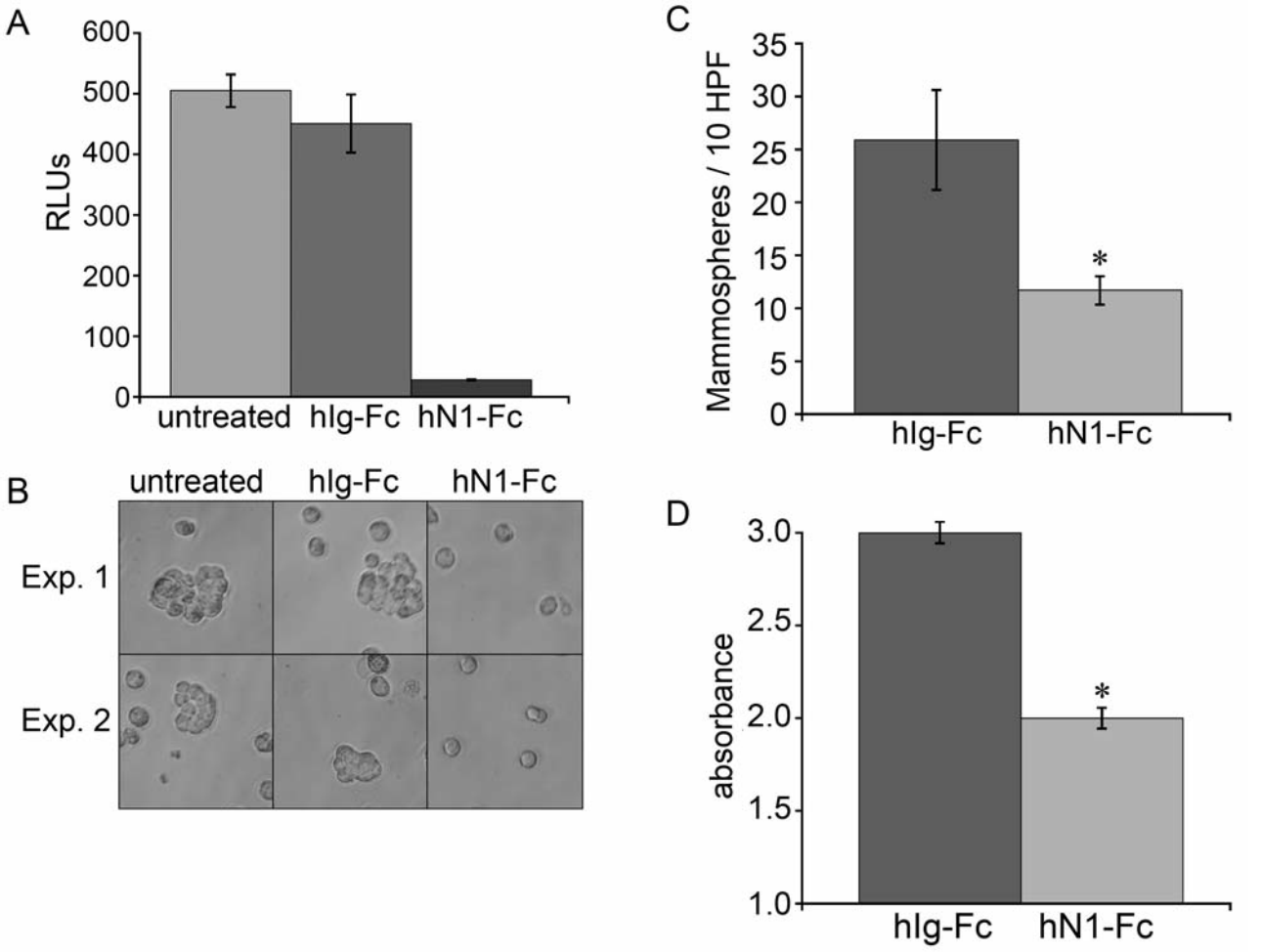

*Figure 8. A Notch decoy protein blocked early sphere formation and proliferation by T47D-A18 cells. A: Luciferase activity in cells transfected with a Notch-responsive luciferase reporter. There was a significant reduction in luciferase activity in cells treated with 3 μM human Notch1-Fc chimera (hN1-Fc), but not 3 μM human IgG1 Fc protein used as a control. B: Representative photographs illustrating the appearance of cells in the experiment. The results are representative of 2 independent experiments. Objective magnification: ×10. C: Quantitation of the results showing a significant reduction in sphere formation in hN1-Fc treated samples. D: The Notch decoy inhibited proliferation of T47D-A18 cells. The results are combined from 2 independent experiments. Statistical analysis was performed using a Student's t-test. *p<0.05.*

elimination of viable cells. The time frame required for cell death could be reduced in these cultures by increasing the MRK003 concentration to 20 μM (data not shown). Moreover, addition of a third dose of drug 96 hours following the second dose also promoted cell killing. On the other hand, 5 μM MRK003 was also able to kill all tested cell lines; however, the time frame for complete killing was extended by several days (data not shown).

Gamma-secretase inhibitors inhibit colony formation in soft agar. Tumor cell lines can be more resistant to pharmacologic agents when grown as 3-dimensional spheroids within a matrix, which more accurately mimics *in vivo* conditions. Therefore, we examined the effect of MRK003 on colony formation in soft agar. T47D-A18 mammospheres were dissociated, and the cells cultured in mammosphere media containing 0.35% agar with or without MRK003. By day 5, small colonies were seen in the DMSO-treated cultures, while MRK003 cultures remained as single cells. At day 10, colonies were counted in 10 random high-powered microscopic fields, which revealed an average of 6.5±0.5 colonies in DMSO-treated *versus* no colonies in MRK-treated cultures (Figure 7A, 7B). By day 14, only cellular debris remained in the MRK-treated samples (Figure 7A). Similar results were obtained in experiments performed with other representative breast cancer cell lines (BT474,

MDA-MB231, Figure 7B). We attempted colony formation assays with two primary ERα-positive pleural effusion samples, but colonies did not form in the untreated samples (data not shown).

Notch decoy protein prevents sphere formation. While gamma-secretases are crucial to Notch activation, they are also involved in intramembranous cleavage of numerous targets including E-cadherin, CD44, and ERBB4 (34). To confirm the role of Notch signaling in mammosphere formation, we utilized a recombinant human Notch-1 Fc chimera protein (hN1-Fc) known to bind Jagged-1 and block Notch activation (35). Initial experiments confirmed blockade of Notch signaling with this decoy using luciferase reporter assays. T47D-A18 cells were transfected with the Hey1 promoter driven luciferase construct, and then cultured in the presence of the hN1-Fc or recombinant human IgG1 Fc (hIg-Fc) as a control. Luciferase activity was unaffected by the hIg-Fc (1.1-fold decrease), but was significantly reduced by hN1-Fc (18-fold decrease, Figure 8A).

Next, established T47D-A18 mammospheres were collected, dissociated, and new cultures initiated in the presence of 3 μM hN1-Fc or hIg-Fc as a control. After 24 hours, small clusters of cells consistent with early mammospheres were apparent in untreated and control-treated cells; while hN1-Fc treated samples remained primarily as single cells (Figure 8B). To quantitate the results, the early mammospheres were counted in 10 random high-powered microscopic fields. The results showed a significant decrease in the number of early mammospheres between hN1-Fc chimera and hIg-Fc control-treated cells (Figure 8B, 8C $p<0.05$). The Notch-1 decoy did not completely block early sphere formation under our experimental conditions. This may be partly due to the fact the decoy was designed to block Notch-1 activity, but it is unknown if it blocks activity of any other Notch receptors. The results were confirmed and extended using an MTT-based proliferation assay. The results showed that hN1-Fc significantly decreased proliferation of mammosphere cultures compared to the hIg-Fc control (Figure 8D, $p<0.05$).

Notch1 siRNA prevents sphere formation. Finally, we used a genetic approach to confirm Notch inhibition is responsible for the observed effects. Initial experiments demonstrated knockdown of Notch-1 expression using pLVTHM-GFP-siNotch1 (siNotch1) in T47D-A18 cells. Western blot analysis showed that Notch-1 was significantly decreased in cells transfected with the siNotch1 construct compared to cells transfected with the pLVTHM-GFP empty vector (siEV) as a control (Figure 9A). Decreased Notch1 expression was not observed after 48 hours (1.2-fold decrease), but was present at 72 hours (2.0-fold decrease) and 96 hours (5.5-fold decrease) after transfection.

Next, T47D-A18 cells were transfected with siNotch1 or siEV, and after 48 hours, a FACS sort used to collect the GFP-positive and GFP-negative cell fractions. Both populations were placed into mammosphere culture, and examined after an additional 48 hours (96 hours after transfection). As anticipated, spheres were evident in the GFP-negative cultures whether they were derived from the siNotch1 or empty vector transfected cells (data not shown). The GFP-positive cultures from siEV-transfected cells also showed evidence of sphere formation (Figure 9B), while the GFP-positive cultures from siNotch1-transfected cells showed no evidence of similar spheres (Figure 9B). Instead, only single, GFP-expressing cells were observed. These results are consistent with our hypothesis that Notch signaling is essential to stem cell self-renewal and supports our findings with both GSIs and the Notch decoy.

Discussion

The CSC hypothesis has recently re-emerged as a compelling theory for tumor formation, recurrence and metastasis (8). Studies have not only identified these cells in primary breast cancer specimens and cell lines, but have also shown they are resistant to killing by standard chemotherapeutics and radiation therapy (7). This has naturally led to questions regarding how the CSC population can be targeted and killed as a means to prevent cancer recurrence. Recent technological advancements have allowed investigators to identify and isolate/enrich for breast CSCs based on their functional activity and phenotypic markers, and tremendous progress has been made in understanding CSC biology from these studies. Serial tumor propagation in orthotopic mouse models remains the gold standard for demonstrating CSC activity (4). However, *in vitro* techniques, such as sphere formation assays and serial colony formation assays, are now routinely used as surrogate assays for self-renewal. Although these assays have limitations, they provide the means to study the biology of the putative CSC (or stem-like cells) and test possible therapeutic compounds prior to initiating long-term *in vivo* studies.

Dysregulation of the Notch signaling pathway has been demonstrated in a variety of tumors, including breast cancer, and studies have shown that Notch inhibition with GSIs can kill breast cancer cell lines *in vitro* and *in vivo* (11, 12). As Notch signal has been implicated in mammary stem cell and breast CSC self-renewal, it is possible GSIs may provide therapeutic benefit by affecting not only bulk tumor cells but also CSCs (10, 17). Since GSIs are in early clinical development in breast cancers, we addressed the questions of whether these agents directly affect the number and/or self-renewal of CSC-like cells, and whether their effects, if any, can be attributed to inhibition of Notch signaling.

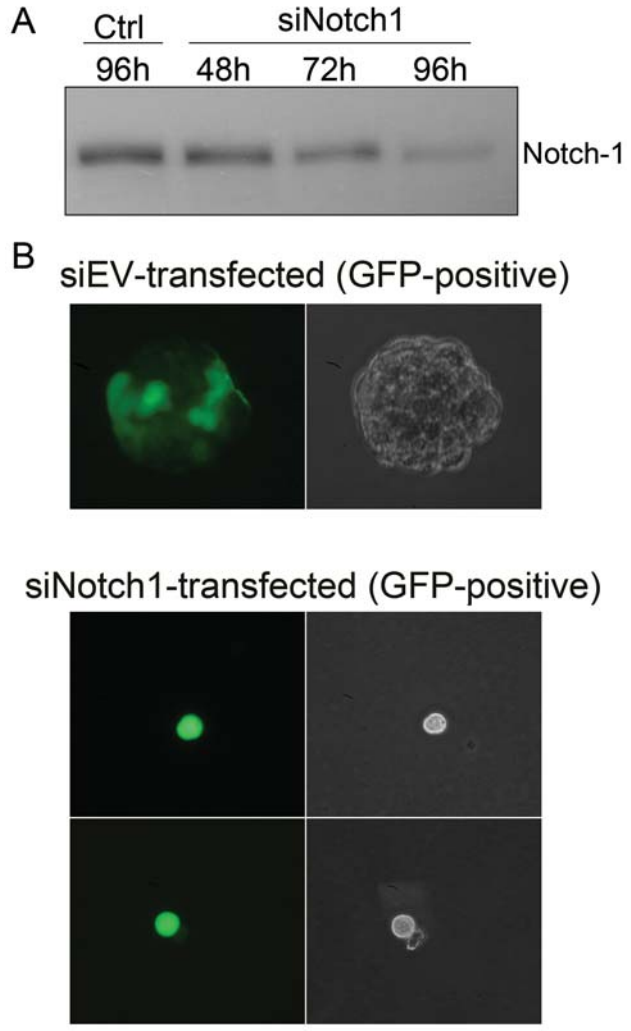

Figure 9. *A Notch1 siRNA inhibits sphere formation. A: T47D-A18 cells transfected with pLVTHM-GFP-siNotch1 (siNotch1) expressed less Notch1 protein at 72 and 96 hours than cells transfected with the empty pLVTHM-GFP vector (siEV) as a control. Data are representative of 3 independent experiments. B: Representative photographs of GFP-positive cells collected from a FACS sort and placed into mammosphere culture. Transfection of siNotch1 blocked mammosphere formation compared to cells transfected with siEV as a control. Data are representative of 2 experiments.*

In the present report, we examined the role of Notch signaling in controlling self-renewal and proliferation of the putative breast CSCs. We found that mammosphere-derived stem and progenitor cells possessed elevated levels of Notch activation as demonstrated by increased expression of immediate downstream targets of Notch signaling, particularly Hes-1, which was elevated in all analyzed samples. Similarly, Hes-1 was increased in mammospheres from two ERα-positive patient samples from metastatic breast cancer each isolated using a different method, supporting the physiological relevance of these findings.

The stem-like cells from each cell line analyzed showed elevated expression of at least one Notch receptor or ligand, but the individual receptors or ligands modulated were different between the cell lines. This variation in expression of Notch pathway components by the different cell lines and patient samples was not unexpected. Notch signaling is known to be strongly context dependent, and multiple factors (such as the Notch ligand/receptors involved, the level of receptor/ligand expression, and the type of cell on which it is expressed) determine which downstream targets are expressed (9, 36). In preliminary microarray experiments (A. Pannuti and L. M., unpublished) we have determined that the genes modulated by selective knockdown of individual Notch receptors and by GSIs are dramatically different in ERα-positive versus ERα-negative cells. In those experiments, we also noted and validated the fact that Hes-1 does not respond well to Notch inhibition in bulk breast cancer cells, while other targets do, particularly in ERα-positive cells (Hao *et al*., submitted for publication). By contrast, in mammospheres from cell lines of different phenotypes and from primary clinical specimens Hes-1 was consistently overexpressed. This suggests that Hes-1 may be a common downstream effector of Notch signaling during the process of progenitor generation and partial differentiation that take place in mammospheres. Thus, Hes-1 may be an appropriate biomarker to follow effects of Notch inhibitors in CSC and CSC-derived mammospheres, but not necessarily in bulk breast cancer cells.

The current report demonstrates that GSIs block sphere formation indicating gamma-secretase activity is essential for maintenance and early differentiation of the stem-like cells. We chose to use GSIs for these studies because these agents are currently in clinical trials, and to determine whether they affect the putative breast CSC has immediate translational relevance. Moreover, GSIs have the advantage of blocking activation of all four Notch receptors. Notch receptors are believed to have partially overlapping functions; hence, inhibition of a single receptor may not always be sufficient to prevent signaling. We evaluated three structurally distinct GSIs at the lowest concentrations of each agent needed to inhibit Notch signaling. This was done to decrease the likelihood of observing Notch-unrelated off-target effects. Moreover, we have confirmed our results with a specific Notch decoy protein that binds Notch ligands and prevents Notch receptor-ligand interactions and a Notch-1 siRNA approach, supporting the observed effect to be Notch dependent.

It has been shown that mammosphere number is a reflection of self-renewal while mammosphere size is related to progenitor cell proliferation (10, 26, 37). Mammosphere formation was significantly decreased in most experiments utilizing LLNle, LY-411,575, or MRK003 suggesting that both self-renewal and proliferation were inhibited, at least

temporarily in the case of LLNle and LY-411,575. It is currently unclear why MRK003 appears to require a slightly longer time period to fully block mammosphere formation, which was particularly apparent in our studies using primary samples from breast cancer pleural effusions. However, we have noted a similar delay in other experimental systems using this compound (KEF, LM unpublished data). Our studies may indicate a functional difference between the different GSIs; however, the divergent results may be related to differences in the potency, solubility, and / or stability of the GSIs. As noted earlier, we have previously reported solubility issues with LY-411,575 (21). Furthermore, both LY-411,575 and LLNle may be more unstable than MRK003. LY-411,575 has a reported half-life of 2-hours *in vivo* and was re-added every 6 hours *in vitro* to maintain Notch inhibition, aldehyde-containing compounds tend to have poor metabolic stability (21, 38). In contrast, MRK003 maintains functional activity *in vitro* for approximately 96 hours. Regardless of the reason, our results indicate GSIs, at least under certain conditions, can induce apoptosis in stem-like cells and mammospheres. This finding may be important for ongoing clinical trials in breast cancer as GSIs may be able to functionally eliminate the CSC population. Mammospheres contain one or two CSC and consist primarily of partially differentiated progenitor cells. Thus, they represent a dynamic environment where proliferation and abortive differentiation are taking place. We showed in 2004 that differentiating cells are particularly sensitive to Notch signaling (39). When Notch-1 was knocked down during pharmacologically induced differentiation of MEL erythroleukemia cells, differentiation was abolished and cells defaulted into apoptosis. Terminal differentiation was accompanied by decreased Notch expression, and constitutive overexpression of activated Notch-1 prevented differentiation and maintained survival. Our results in mammospheres may represent a similar phenomenon, suggesting a model in which when CSCs initiate differentiation within a mammosphere, they require Notch signaling (and presumably Hes-1) for survival. When Notch is inhibited, differentiating CSC default into apoptosis. This may not be the same mechanism of action underlying growth arrest and cytotoxicity induced by Notch inhibitors in bulk breast cancer cells.

A few studies have begun to address Notch signaling in mammary stem cell or breast CSC. Dontu *et al*. performed a comprehensive analysis of Notch signaling in normal mammary stem cells (10). They demonstrated a 10-fold increase in secondary mammosphere formation and mammosphere size when Notch was activated using exogenous ligand, and found an increase in myoepithelial cell progenitors or in their proliferation (10). In contrast, Notch inhibition, using either a Notch-4 neutralizing antibody or GSI, effectively blocked mammosphere formation; however, the inhibitors had no effect if added after mammospheres had already formed (10). They concluded that Notch activation played a role in the initial stages of mammosphere formation; and our results are consistent with and expand their findings. In regards to breast CSCs, Farnie *et al*. blocked Notch signaling in mammospheres derived from DCIS using Notch-4 neutralizing antibodies or DAPT, another GSI. They found that Notch inhibition blocked mammosphere forming efficiency, but indicated the Notch-4 antibody was more effective than the GSI (17). This may indicate that Notch-4 is particularly important in self-renewal of the CSCs involved in DCIS, and this group recently followed up with a new study indicating Notch-4 is preferentially activated in CSCs (20). Finally, recent studies by Sansone *et al*. indicate a potentially important role for Notch-3 in stem/progenitor cells cultured as mammosphere, which mimics a mildly hypoxic environment (18, 19). They reported that IL-6 induced Notch-3 and Jagged-1 expression in mammospheres derived from MCF7 or primary breast CSC and promoted mammosphere growth, which was inhibited if either IL-6 or Notch-3 signaling was blocked. Their results show a marked reduction in mammosphere size indicating an important role for Notch-3 in progenitor proliferation.

Recent studies have implicated other signaling pathways in the maintenance of mammary stem / progenitor cells and/or breast CSC. Of particular interest, Zhou *et al*. isolated stem-like cells from the MCF7 cell line, and showed that NF-κB inhibitors reduced the SP and blocked mammosphere formation (40). Although there was no difference in basal levels of NF-κB activity in the stem-like cells and bulk cultured MCF7 cells, the inhibitors appeared more effective in blocking NF-κB signaling in the mammospheres than the bulk cells. Numerous reports have linked NF-κB and Notch signaling, and have demonstrated that Notch can either promote or inhibit NF-κB activity (and *vice versa*) in a context-dependent manner (41). It is possible that modulation of NF-κB activity in the MCF7 cells inhibited Notch activity resulting in reduced mammosphere formation or vice versa. Additional studies will be necessary to determine if crosstalk between the Notch and NF-κB pathways occurs in the putative CSC.

In summary, the current report demonstrates that stem-like cells derived from ERα-positive, -negative and Her2/neu overexpressing breast cancer cell lines express elevated levels of the Notch target gene, Hes-1, as well as mRNA from various other Notch targets, receptors and ligands. Similar results were obtained in with two primary samples from ERα-positive pleural effusions. Treatment of cells with any of three structurally distinct GSIs significantly decreased mammosphere formation, which was confirmed in T47D-A18 cells with a specific Notch decoy protein and Notch-1 siRNA, further supporting a role for Notch in stem cell self-renewal. Importantly, mammosphere formation was irreversibly eliminated in cells treated with MRK003, but not

in cells treated with either LLNle or Ly-411,575 where the effect was transient. These novel 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.

Acknowledgements

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