



# Inhibition of Notch signaling reduces the stem-like cell population in T47D breast cancer cells and prevents mammosphere formation

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Abstract #106

## Abstract

Breast cancer has been proposed as a stem cell-derived disorder based on studies identifying cancer stem cells (CSCs) in breast tumors and established cell lines. These self-renewing CSCs may be 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, can be effectively blocked by  $\gamma$ -secretase inhibitors (GSIs). While GSIs are currently in clinical trials for breast cancer, it is unclear how these compounds will affect CSCs or if CSCs from different breast cancer phenotypes (estrogen receptor- $\alpha$  (ER)-positive and negative) will be differentially affected. Here, we demonstrate stem-like cells derived from the T47D breast cancer cell line show elevated levels of Notch signaling, with an ER- subclone (T47D-C42) having higher intrinsic levels of Notch activity compared to an ER+ subclone (T47D-A18). Blockade of Notch signaling with three structurally distinct GSIs reduced the number of stem-like cells, and completely abolished secondary mammosphere formation, which is a measure of stem cell self-renewal. Importantly, this effect was irreversible with MRK-003, but not the other GSIs. Moreover, our results suggest the ER- cell line was more sensitive to the GSIs compared to the ER+ line. This is consistent with our recent report that loss of estrogen signals results in Notch activation. 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.

## Background

- Cancers may originate from a subset of tumor cells referred to as CSCs.
- These cells possess key properties of normal stem cells including the ability to self-renew and resistance to chemical insults.
- Standard therapies may effectively kill most tumor cells, but CSCs may persist and eventually cause relapsed disease.
- The Notch signaling pathway consists of four Notch receptors (Notch1-4) and five ligands (Jagged-1,2; Dll-1, 3, 4). Notch activation requires cleavage of the receptor by  $\gamma$ -secretase and is blocked by  $\gamma$ -secretase inhibitors.
- Notch signaling is a mediator of mammary gland development and has been implicated in mammary stem cell self-renewal and differentiation.
- Deregulated Notch signaling has been identified in breast cancer and is associated with a poor prognosis.

## Objectives

- Evaluate Notch expression and activation in stem-like cells derived from two subclones of the T47D cell line: T47D-C42 (ER-) and T47D-A18 (ER+).
- Determine if Notch inhibition alters breast CSC self-renewal and proliferation

### Results I. Notch expression / activation is elevated in stem-like breast cancer cells.

- Side population (SP) cells are a heterogeneous subset of cells known to be enriched for stem cells.
- SP cells can be readily isolated from the T47D-C42 (ER-) and T47D-A18 (ER+) breast cancer cell lines (not shown).
- SP cells expressed elevated mRNA levels of Notch receptors (Notch-1, -4) and downstream targets of Notch (Hes-1, Hey-1) compared to non-SP cells (Figure 1). Hes-1 and/or Hey-1 expression indicate Notch activation.

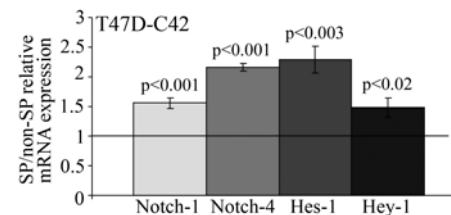


Fig 1. Quantitative RT-PCR showed elevated Notch receptor and target gene expression in SP compared to non-SP cells in T47D-C42 cells. The same pattern was found in T47D-A18 cells, but the results were not significant (not shown). Relative mRNA expression was calculated using the comparative method where expression in non-SP cells was set to 1, and SP cell expression graphed.

### Results II. $\gamma$ -secretase inhibitors (GSIs) reduce the SP and inhibit mammosphere formation in T47D breast cancer cells

- Treatment of bulk T47D-C42 or T47D-A18 cells with any of three different GSIs (LLNle, MRK003, LY-411,575 - all known Notch inhibitors) significantly reduced the percentage of cells in the SP (Figure 2).
- Mammospheres were readily formed from T47D-C42 and T47D-A18 cells. The spheres were characterized with immunostaining and RT-qPCR for known stem / progenitor cell markers (not shown).
- Secondary mammosphere formation was abolished by GSIs at the 48 hour time point (Figure 3, 4).
- The results were reversible with two of the GSIs, LLNle and LY-411,575, as mammospheres reformed at approximately 5 days. However, the results were irreversible with MRK-003 (Figure 3, 4). After 7 days, the remaining cells appeared non-refractile, condensed, and fragmented.

## Results

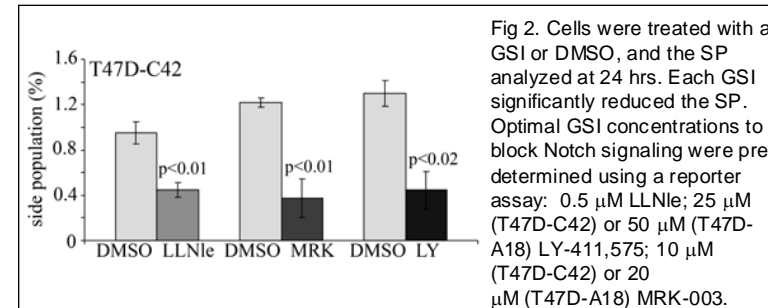


Fig 2. Cells were treated with a GSI or DMSO, and the SP analyzed at 24 hrs. Each GSI significantly reduced the SP. Optimal GSI concentrations to block Notch signaling were pre-determined using a reporter assay: 0.5  $\mu$ M LLNle; 25  $\mu$ M (T47D-C42) or 50  $\mu$ M (T47D-A18) LY-411,575; 10  $\mu$ M (T47D-C42) or 20  $\mu$ M (T47D-A18) MRK-003.

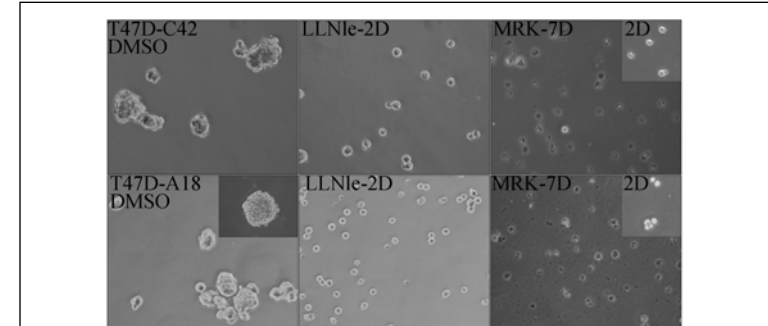


Fig 3. Mammospheres were readily detected in DMSO-treated cultures at 2 days (2D), while LLNle or MRK-treated cultures showed primarily single cells. With LLNle and LY, the results were temporary, and spheres reformed after 5-7 days (not shown). In contrast, MRK treatment was irreversible, and after 7 days (7D), the cells appeared non-refractile, condensed, and fragmented.

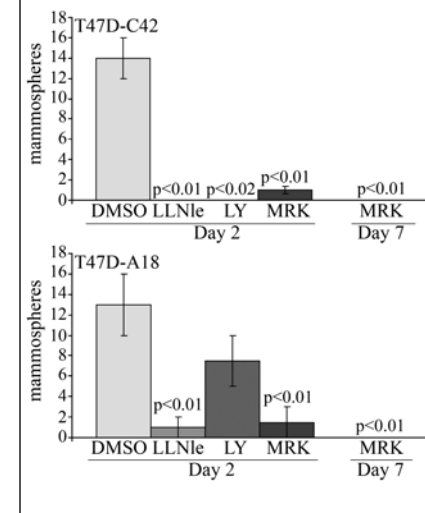


Fig 4. Quantitation of sphere formation. Each GSI blocked sphere formation at 2 days. With LLNle and LY, spheres reformed (not shown), but MRK abolished sphere formation irreversibly. Even after 10 days, no evidence of cell proliferation was found in either MRK-treated T47D-A18 or T47D-C42 cells.

### Results III. Blockade of Notch signaling with a specific Notch decoy protein inhibits mammosphere formation.

- A specific Notch decoy protein known to inhibit ligand-receptor interactions blocked sphere formation while a control recombinant protein had no effect.
- The results confirm a role for Notch signaling in mammosphere formation.

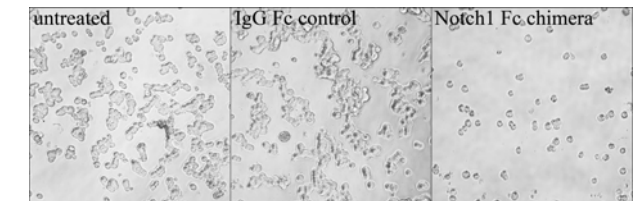


Fig 5. To demonstrate Notch inhibition was responsible for the results, T47D-C42 cells were treated for 24 hrs with a recombinant human Notch1 Fc chimera. Secondary mammosphere formation was significantly inhibited by the Notch decoy (3  $\mu$ M), but not a human IgG Fc protein used as a control (3  $\mu$ M).

## Conclusion

- GSIs are in early clinical development for breast cancer, but their affect on CSCs is unknown.
- Studies have shown breast cancer cell lines are excellent models for studying CSCs as they maintain the hierarchical cellular heterogeneity characteristic of primary tumors. We chose two well-characterized subclones of T47D cells representing ER- and ER+ phenotypes.
- The ER- subclone appeared more sensitive to GSIs, which is consistent with the higher levels of Notch activity seen in these cells with RT-qPCR.
- Mammosphere formation was irreversibly eliminated in cells treated with MRK-003, but was temporary in cultures treated with LLNle or LY-411,575. The results suggest MRK-treated cells were dead or permanently growth arrested.
- Our findings support a role for Notch signaling in CSC self-renewal and proliferation, and suggest Notch inhibition may have clinical benefits in targeting CSCs.

## Disclosure

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