

Regulation of Breast Cancer Stem Cell Activity by Signaling through the Notch4 Receptor

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Abstract

Notch receptor signaling pathways play an important role not only in normal breast development but also in breast cancer development and progression. We assessed the role of Notch receptors in stem cell activity in breast cancer cell lines and nine primary human tumor samples. Stem cells were enriched by selection of anoikis-resistant cells or cells expressing the membrane phenotype $ESA^+/CD44^+/CD24^{low}$. Using these breast cancer stem cell populations, we compared the activation status of Notch receptors with the status in luminally differentiated cells, and we evaluated the consequences of pathway inhibition *in vitro* and *in vivo*. We found that Notch4 signaling activity was 8-fold higher in stem cell-enriched cell populations compared with differentiated cells, whereas Notch1 signaling activity was 4-fold lower in the stem cell-enriched cell populations. Pharmacologic or genetic inhibition of Notch1 or Notch4 reduced stem cell activity *in vitro* and reduced tumor formation *in vivo*, but Notch4 inhibition produced a more robust effect with a complete inhibition of tumor initiation observed. Our findings suggest that Notch4-targeted therapies will be more effective than targeting Notch1 in suppressing breast cancer recurrence, as it is initiated by breast cancer stem cells. *Cancer Res*; 70(2); 709–18. ©2010 AACR.

Introduction

Identification of cell surface markers has allowed the enrichment of cancer stem cells (CSC) from the total cell population in leukemia (1) and several solid tumors, including prostate, colorectal, and brain (2–4). In the breast, CSCs are enriched by sorting for $ESA^+/CD44^+/CD24^{low}$ and this population is tumor initiating *in vivo* (5).

Comparisons between $CD44^+$ and $CD24^+$ breast cancer cells from patient samples have shown that the breast $CD44^+$ cells are basal like, similar to normal breast stem cells. In contrast, $CD24^+$ cells express markers of luminal differentiation (6). These two cell types, the basal $CD44^+$ breast CSCs (BCSC) and the luminally differentiated $CD24^+$ cells, have

also been shown to exist in a breast cancer cell lines (7), indicating a similar cellular hierarchy to primary breast cancer tissue.

In addition to cell surface phenotype, suspension mammosphere culture can be used to study normal and CSCs *in vitro*. This has been successfully used to grow colonies from stem cells in nonadherent culture and to measure the capacity of breast cells to self-renew and produce differentiated progeny, two known characteristics of normal stem cells and CSCs (8–10).

The resistance of BCSCs to treatment has been shown by studying breast cancer cells taken from patients before and after neoadjuvant chemotherapy. An increased proportion of $CD44^+/CD24^{low}$ breast cancer cells was observed after treatment, suggesting that BCSCs were able to preferentially survive treatment compared with the more differentiated cancer cells (11). The Notch pathway has also been linked to radiation resistance in breast cancer cell lines; BCSCs, isolated by nonadherent culture, have increased resistance to radiation compared with the more differentiated nonstem cells and Notch signaling is increased in these cells (12).

The Notch signaling pathway plays an important role in normal breast development, cell fate, and stem cell self-renewal (13), and its deregulation has been shown to play a role in cancer. A role for Notch was first identified in mouse mammary tumors, which had frequent integration of the mouse mammary tumor virus (MMTV) into the Notch4 receptor, resulting in a truncated intracellular form of the receptor that is constitutively activated (14).

Aberrant Notch signaling has been implicated in the development and progression of both preinvasive ductal carcinoma *in situ* (DCIS; ref. 10) and invasive breast cancer (15, 16).

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Overexpression of Notch receptors has been reported in both DCIS (10) and invasive cancer (16). Furthermore, high levels of ligands (17–19), downstream targets (16), as well as down-regulation of Numb (15) have been reported in invasive breast cancer.

Although Notch signaling is clearly important in the development and progression of breast cancer, little is known about its activity in the BCSC subpopulation. We show that BCSC activity depends preferentially on Notch4, rather than Notch1, receptor signaling. This improved knowledge of the role of Notch signaling in BCSCs will allow the design of more successful breast cancer treatments.

Materials and Methods

Primary cell isolation. Pleural effusion samples from patients with metastatic breast cancer ($n = 7$) and primary solid tumor samples ($n = 2$) were collected with fully informed consent (COREC# 05/Q1402/25 and 05/Q1403/159). For details, see Supplementary Table S1. Pleural effusion cells were harvested as previously described (20). Solid tumors were cut into ≤ 1 -cm pieces and disaggregated in complete medium with 12 \times 1-min compressions using the Stomacher 80 Biomaster (Seward). Remaining leukocytes were removed with CD45-negative magnetic sorting according to the manufacturer's instructions (Miltenyi Biotech).

Monolayer and mammosphere culture. Monolayers of the human breast cancer cell lines were grown as previously described (21). Monolayer cells were enzymatically [0.125% trypsin-EDTA (Sigma)] and manually (25-gauge needle) disaggregated to a single-cell suspension. Primary cells were resuspended as single cells in PBS. Cells were plated at 500/cm² in nonadherent culture as previously (10), and flasks were coated in 1.2% poly(2-hydroxyethylmethacrylate)/95% ethanol (Sigma). Cells were grown for 7 d in DMEM/F12 containing B27 and MEGM SingleQuots (human epidermal growth factor, insulin, hydrocortisone, and GA-1000; Cambrex) and maintained in a humidified incubator at 37°C at an atmospheric pressure in 5% (v/v) carbon dioxide/air. Percentage mammosphere-forming units (%MFU) were calculated as number of mammospheres (≥ 50 μ m) formed divided by the cell number plated and multiplied by a hundred.

Viable cell count. Annexin/propidium iodide staining was carried out according to the manufacturer's instructions (Apoptosis Detection Kit I, BD Biosciences). Staining was assessed using the Becton Dickinson FACSCalibur, and levels were analyzed using WinMIDI 2.8.

Flow cytometric analysis and sorting. Cells were resuspended at $\leq 1 \times 10^6$ in 100 μ L sorting buffer (PBS containing 0.5% bovine serum albumin, 2 mmol/L EDTA) and incubated with pre-conjugated primary antibodies BERE4-FITC (1:10; Dako), CD44-APC (1:20; BD Pharmingen), and CD24-PE (1:10; Beckman Coulter) for 10 min at 4°C. The cells were washed in PBS and centrifuged at 800 \times g for 2 min. For analysis, cells were resuspended in 500 μ L of sorting buffer and fluorescence was measured using FACSCalibur and analyzed using WinMIDI 2.8. For sorting, cells were resuspended in 1 \times HBSS (Invitrogen) after incubation with the primary

antibodies. Cells were sorted, with HBSS as sheath fluid, at 16 p.s.i. using FACS Aria. The CD24^{low} cell population gated by FACS was the lowest quintile of CD24-positive cells plus all the CD24-negative cells.

Immunoblot analysis. Western blotting was carried out as previously described (10, 21). For details of antibodies used, see Supplementary Table S2. Densitometry was performed using ImageJ software freely available online.⁷ Mean band intensity was measured (minus background intensity) and fold change from actin control was calculated.

In vitro inhibition of the Notch pathway. For γ -secretase inhibition (GSI) of signaling, 10 μ mol/L of the GSI, DAPT {*N*-[*N*-(3,5-difluorophenacetyl-L-alanyl)]-*S*-phenylglycine *t*-butyl ester; Calbiochem}, was added to monolayer or mammosphere culture at the day of plating (final DMSO concentration 0.01%). Dibenzazepine (DBZ; 10 μ mol/L) in DBZ buffer (0.5% Methocel, 0.1% Tween 80) was used to treat MCF7 in adherent culture for 3 d for Western blot analysis of signaling inhibition.

Pre-designed small interfering RNA (siRNA) sequences were acquired from Dharmacon to target four unique sequences in the Notch1 and Notch4 receptors (for sequence details, see Supplementary Table S3). MCF7 cells were transfected according to the manufacturer's instructions.

Production of overexpressing and knockdown cell lines. MDA-MB-231_Numb cell production was previously described by Stylianou and colleagues (16). To produce inducible Notch1 intracellular domain (N1-ICD) cells, MCF7 and MDA-MB-231 cells were transduced with p201-doubleTet lentivirus in the presence of 8 μ g/mL polybrene and selected with 1 and 1.5 mg/mL of G418, respectively. DoubleTet lines were then infected with lentivirus (p199-YN1-ICD-iTK-SVzeo) and then selected with 1.5 mg/mL zeocin (Sigma).

Doxycycline-inducible stable short hairpin RNA (shRNA) cell lines were produced using the Clontech pSingle-tTS-shRNA vector for the Notch1 and Notch4 receptors and a scrambled control according to the manufacturer's instructions. Cell lines were grown in DMEM containing 10% tetracycline-free fetal bovine serum (Biosera), L-glutamine, antibiotics, and 0.5 μ g/mL G418 (Life Technologies).

In vivo limiting dilution and Notch inhibition. All procedures were performed in accordance with the Animals (Scientific Procedures) Act 1986 and approved by the UK Home Office. Cell lines were resuspended in 0.2 mL PBS and injected s.c. into mice treated with estrogen pellets (0.72 mg; Innovative Research of America).

For inducible shRNA, knockout mice were given 2 mg/mL doxycycline (Sigma) and 5% sucrose (Sigma) in their drinking water from the day of cell injection, and control mice were given sucrose only. For GSI, 1 mg/mL DBZ (a kind gift from Adrian Harris, Weatherall Institute of Molecular Medicine, Oxford, UK) was delivered by i.p. injection on the day of cell injection and every subsequent 3 d.

Statistical methods. Throughout the article, data are represented as mean \pm SE taken over a minimum of three

⁷ <http://rsb.info.nih.gov/ij/>

independent experiments, unless otherwise stated. Statistical significance was measured using parametric testing, assuming equal variance, in the majority of experiments with standard *t* tests for two paired samples used to assess difference between test and control samples. Two-way ANOVA was used to assess difference in tumor formation *in vivo*.

Results

Identification of BCSCs in cell lines and primary samples. We evaluated *in vitro* mammosphere culture of breast cancer cell lines, invasive primary tumors and malignant pleural effusions from patients. Mammospheres formed in all cell types tested (Fig. 1A). Formation rate varied considerably but did not correlate with grade or steroid receptor status (Supplementary Table S1). Importantly, we showed that mammospheres formed from single cells (Supplementary Fig. S1A) contained a single-label retaining cell in $\geq 80\%$ of cases (Supplementary Fig. S1B and C) and a single MFU (Supplementary Fig. S1D), indicating their clonal origin.

At early time points in nonadherent culture, we predicted that MFU would be enriched because differentiated cells undergo anoikis in these conditions (8). Eighty-five percent of MCF7 cells underwent anoikis within 16 hours of culture (Fig. 1B; $P < 0.05$). The anoikis-resistant cells, collected at 16 hours, are significantly enriched for *in vitro* MFU (5.7-fold, $P < 0.001$; Fig. 1C). *In vivo*, 50% tumor formation required 5×10^4 anoikis-resistant cells, whereas 6×10^5 monolayer cells are required for the same level of tumor formation, suggesting a 12-fold enrichment for tumor-initiating cells (Fig. 1D). When anoikis-resistant tumors were collected and cells were passaged *in vivo*, all could initiate secondary tumor formation (data not shown), which shows self-renewal (22). These results show that anoikis-resistant cells are enriched for MFU and tumor-initiating cells and suggest, but do not formally prove, that these are overlapping cell populations that are enriched for self-renewing BCSCs.

Isolation of BCSCs. The cell surface phenotype of $ESA^+/CD44^+/CD24^{low}$ isolates cells that initiate tumors (5) and mammosphere culture enriches for the cells *in vitro* (9). CD24 is a marker of differentiated luminal cells, and cells with low expression of this marker are basal myoepithelial cells in the normal mammary epithelium (23). Flow cytometric analysis of MCF7 cells shows that they range from the basal CD44⁺ population to a more differentiated luminal CD44⁻/CD24⁺ population (Fig. 2A). Anoikis-resistant MCF7 cells are significantly enriched for $ESA^+/CD44^+/CD24^{low}$ cells, with 70% expressing this surface phenotype compared with 6% in monolayer cells (Fig. 2A; $P < 0.02$).

Breast cancer cell lines and primary cells collected from pleural effusion samples were sorted using fluorescence-activated cell sorting into four populations (P) based on their cell surface phenotype. Cells were classified as the putatively BCSC-enriched $ESA^+/CD44^+/CD24^{low}$ population (P1), $ESA^+/CD44^+/CD24^{low}$ (P2), $ESA^+/CD44^+/CD24^+$ (P3), or $ESA^+/CD44^-/CD24^+$ (P4). The BCSC-enriched cells (P1) and BCSC-depleted cells (P2–P4) were plated in mammosphere culture to assess MFU enrichment. P1 showed an enrichment for

MFU in all cell types tested (MCF7, 12-fold; T47D, 4-fold; MDA-MB-231, 2-fold; BT474, 5-fold; and primary breast cancer samples, 5.4-fold) compared with other cell subpopulations (Fig. 2B; $P < 0.01$). Mice injected with 1×10^4 P1 MCF7 cells formed subcutaneous tumors in 75% of cases (Fig. 2B; $n = 4$), whereas up to 3×10^6 P2 to P4 MCF7 cells resulted in no tumor growth, which is supportive of previously published work (5). When unsorted MCF7 cells were injected, $>1 \times 10^6$ were required to form tumors with a 75% take rate (Fig. 2B), suggesting a >100 -fold enrichment for tumor-initiating cells in sorted P1 cells. These data suggest the putative BCSC subpopulation is essential for tumor initiation and growth.

Notch receptors are differentially activated in $ESA^+/CD44^+/CD24^{low}$ cells. Next, we examined Notch activation in BCSCs. Notch receptor and ligand expression are differentially expressed in breast cancer, particularly Notch1 and Jagged1 (19). Previously published data have studied protein expression in the whole-cell population comparing cancer with the normal breast (16). In contrast, we aimed to look specifically at the activity of the Notch signaling pathway in BCSCs.

To do this, we examined the differential activity of Notch1 and Notch4 receptors in sorted MCF7 and MDA-MB-231 cells and primary breast cancer samples (Fig. 2C). The antibody to cleaved Notch1 recognized the Notch1 extracellular truncation (Notch4-EXT; *top band*) and the N1-ICD (*bottom band*). A significantly lower level of the cleaved N1-ICD was seen in the BCSC-enriched MCF7 cells (P1) compared with the other subpopulations. N1-ICD levels in P2, P3, and P4 were 3.5 ± 1.2 -fold, 4.1 ± 1.3 -fold, and 3.4 ± 1.5 -fold higher, respectively, than in P1 (Fig. 2C; $P < 0.05$), suggesting lower signaling through Notch1 receptor in BCSC. In contrast, significantly higher levels (up to 20-fold) of the active cleaved Notch4 ICD (N4-ICD) are detected in the BCSCs (P1; $P < 0.05$), suggesting strong signaling through this receptor (Fig. 2C). A similar pattern of Notch1 and Notch4 activation in differentiated versus stem cell-enriched populations, respectively, was observed in MDA-MB-231 and primary breast cancer samples (Fig. 2C).

To confirm Notch1 and Notch4 activation patterns *in vivo*, immunohistochemical analysis of normal and malignant breast samples was performed using antibodies recognizing cleaved N1-ICD and N4-ICD. In normal epithelium, the basal cell layer had higher nuclear expression of N4-ICD, whereas nuclear N1-ICD was detected most strongly in the luminal cell layer (Fig. 2D). In invasive breast cancers, N1-ICD was present in the majority of cells, whereas N4-ICD was more infrequent and strongly stained the nuclei of invasive cancer cells (Fig. 2D). These observations corroborate the pattern of signaling seen in MCF7 and primary breast cancer cells sorted for basal and luminal cell surface markers.

Notch inhibition reduces BCSC number and activity *in vitro* and *in vivo*. Next, we aimed to target Notch signaling and test its effects on BCSCs. One method of Notch inhibition is the use of GSIs, such as DAPT, DBZ, and MK-0752, which stop Notch-ICD cleavage and its subsequent nuclear translocation. DAPT has previously been shown, *in vitro*, to

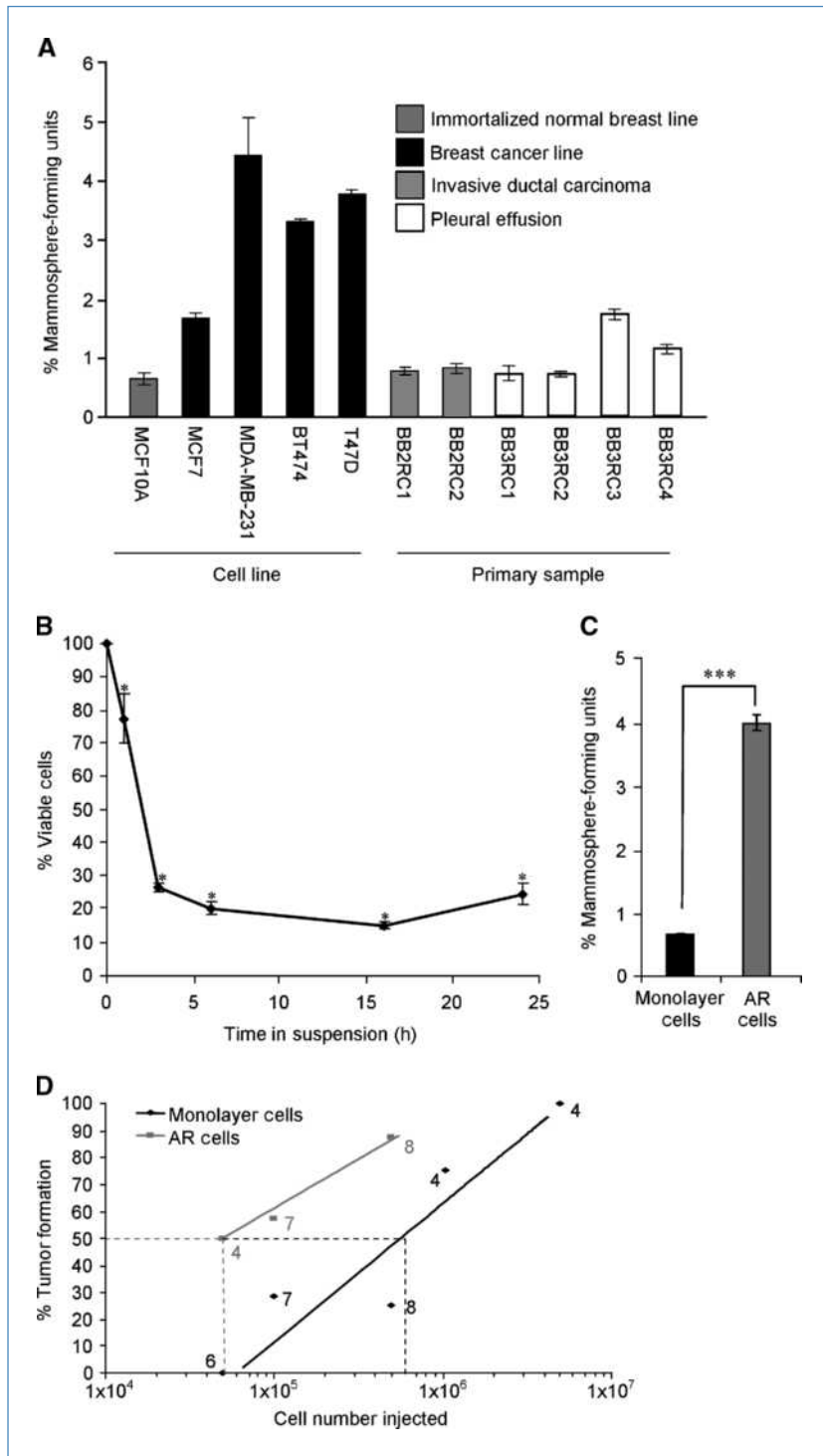


Figure 1. A, percentage MFUs in different cell types. B, MCF7 cell viability assessed using Annexin/propidium iodide staining and analyzed using FACSCalibur after a time course of nonadherent culture. C, MFU number in anoikis-resistant (AR) cells collected at 16 h. D, percentage tumor formation of anoikis-resistant and monolayer cells. Number, mice in group; dotted line, 50% formation. *, $P < 0.05$, compared with 0-h control; ***, $P < 0.001$. A to C, columns and points, mean; bars, SE.

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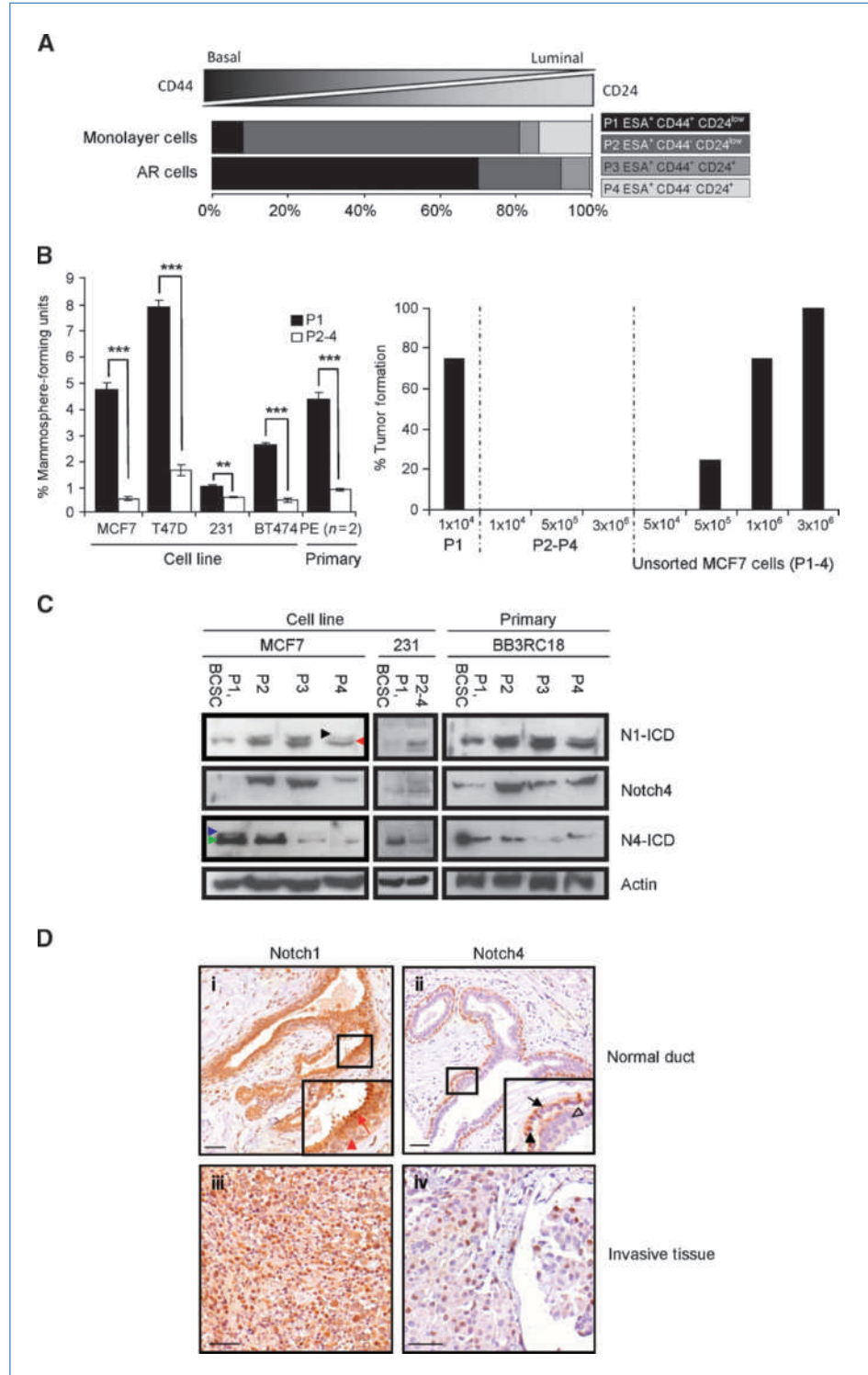
decrease the formation of normal and DCIS mammospheres (10, 13), and MK-0752 is currently being tested in patients (24). We therefore examined the effect of DAPT, DBZ, and specific Notch receptor knockdown on BCSC activity in MCF7 and MDA-MB-231 cells and primary breast cancer samples.

In vitro DAPT (10 $\mu\text{mol/L}$) treatment of MCF7 monolayer cells caused a 30% decrease in the proportion of $\text{ESA}^+/\text{CD44}^+/\text{CD24}^{\text{low}}$ cells (Fig. 3A; $P < 0.01$). However, Notch4 but not Notch1 siRNA reduced the numbers of $\text{ESA}^+/\text{CD44}^+/\text{CD24}^{\text{low}}$ cells (Fig. 3A; $P < 0.05$), suggesting that DAPT affects this population through inhibition of other Notch receptors. In MCF7

and MDA-MB-231 cells, primary invasive ductal carcinoma, and pleural effusion samples, a decrease in MFU was observed (50% decrease in MCF7 and MDA-MB-231 and 30% decrease in pleural effusion and invasive ductal carcinoma samples; Fig. 3B; $P < 0.05$). A corresponding decrease in Notch downstream

target genes, *HES1* and *HEY2*, was seen in the whole-cell population (data not shown). No significant reduction in mammosphere formation was seen in cells treated with DAPT when N1-ICD was activated, suggesting that GSI specifically blocked the effects of Notch receptor signaling (Fig. 3C).

Figure 2. A, anoikis-resistant and monolayer MCF7 cells were analyzed for cell surface phenotypes of breast cancer cells ranging from basal-like BCSCs (CD44⁺/CD24^{low}) to more differentiated luminal cells (CD44⁻/CD24⁺) using FACSCalibur. MCF7, T47D, MDA-MB-231, BT474, and primary pleural effusion cells were sorted (P1, BCSC-enriched ESA⁺/CD44⁺/CD24^{low}; P2, ESA⁺/CD44⁻/CD24^{low}; P3, ESA⁺/CD44⁺/CD24⁺; and P4, ESA⁺/CD44⁻/CD24⁺) and plated in mammosphere culture. ESA, epithelial-specific antigen. B, percentage MFU number was calculated at day 7 in BCSC-enriched cells (P1) and BCSC-depleted cells (P2-4). Percentage tumor formation measured over 5 wk in mice injected with a limiting dilution of sorted and unsorted MCF7 cells. Columns, mean; bars, SE. **, $P < 0.01$; ***, $P < 0.001$. C, immunoblots for full-length and cleaved Notch receptors (N1-ICD, Notch4, and N4-ICD) in sorted cell populations from MCF7 and MDA-MB-231 cell lines and a primary pleural effusion (PE) sample. Black arrowhead, Notch4-EXT; red arrowhead, N1-ICD; blue and green arrowheads, Notch4-EXT and N4-ICD, respectively. D, representative photomicrographs of breast tissue sections stained with antibodies to Notch receptors. Cleaved Notch1 (N1-ICD) expression was assessed in normal (i; red arrow, highly positive nucleus in luminal cell; red arrowhead, weak staining in basal cell nucleus) and invasive tumor tissue (iii). N4-ICD staining in normal (ii; black arrow, inactive cytoplasmic staining; filled black arrowhead, positive nucleus; hollow black arrowhead, cell negative for the stain) and invasive tumor samples (iv). Scale bars, 100 μ m.



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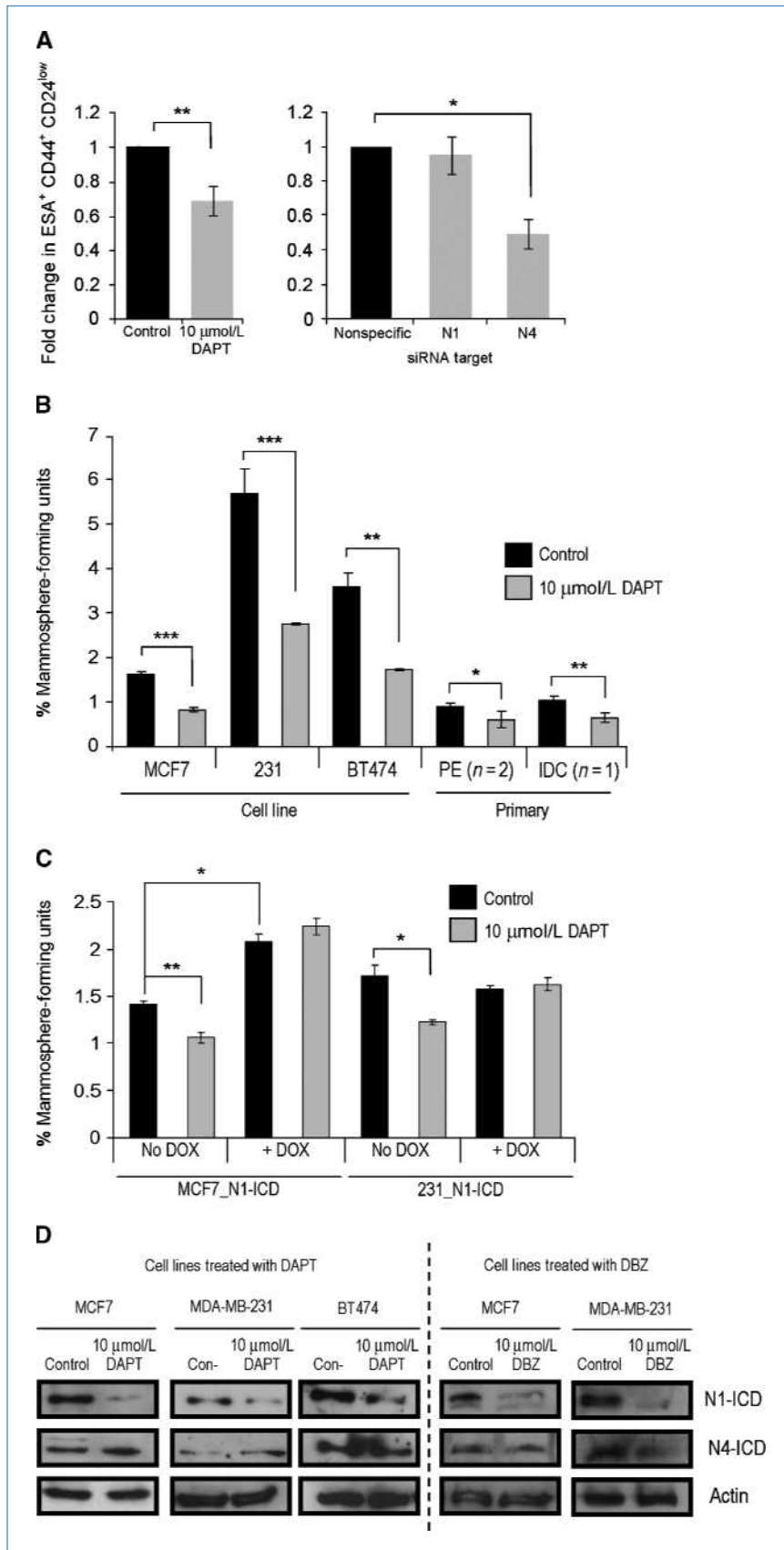


Figure 3. A, ESA⁺/CD44⁺/CD24^{low} cells assessed *in vitro* following Notch inhibition of MCF7 cells with GSI, 10 μmol/L DAPT, or DMSO control and siRNA to Notch1 and Notch4. B, percentage MFU number was assessed in MCF7, MDA-MB-231, and BT474 cells, pleural effusion, and primary invasive ductal carcinoma (IDC) samples (BB3RC2, BB3RC3, and BB2RC2) after 7 d in nonadherent culture with/without DAPT. C, cell lines were produced with doxycycline (DOX)-inducible expression of N1-ICD. These lines, MCF7_N1-ICD and MDA-MB-231_N1-ICD, were cultured with/without DAPT either in the absence (control) or in the presence (activated) of doxycycline. A to C, columns, mean; bars, SE. *, *P* = 0.05; **, *P* < 0.01; ***, *P* < 0.001. D, immunoblot of cleaved Notch receptors (N1-ICD and N4-ICD) was carried out in MCF7, BT474, and MDA-MB-231 cells with or without DAPT treatment and in MCF7 and MDA-MB-231 with or without DBZ.

We then examined the effects of a GSI DBZ on tumor inhibition *in vivo*. DBZ significantly decreased MCF7 but not MDA-MB-231 tumors (Table 1; $P = 0.02$) and increased latency compared with control mice (18–28 days). DBZ-treated MCF7 tumors that did form had significantly reduced tumor volumes ($P = 0.03$). To validate inhibition of Notch signaling, DAPT and DBZ effects on N1-ICD and N4-ICD were measured in MCF7, BT474, and MDA-MB-231 cells. Both inhibitors caused marked reductions in N1-ICD but had no effect on N4-ICD levels in three cell lines examined (Fig. 3D). This suggests a specific or preferential inhibitory effect on the cleavage of the Notch1 receptor at this concentration. This differential effect of GSI on Notch receptors could allow continued activity of Notch4 in the BCSCs and, thus, initiation and maintenance of tumors. To assess the effect of inhibition of all Notch receptor activity, MDA-MB-231 cells overexpressing Numb, the Notch inhibitor, were transplanted into mice. Tumor initiation was completely ablated, whereas vector control cells grew tumors (Table 1), suggesting that inhibition of multiple Notch receptors has a more profound effect than GSI.

Notch4 signaling has a greater effect on BCSC activity than Notch1. Because DAPT and DBZ preferentially affect Notch1 activity, we compared the effect of Notch1- and Notch4-specific knockdown (using RNA interference) on mammosphere and tumor formation. Doxycycline-inducible shRNA MCF7 cell lines targeting each receptor (MCF7^{Notch1} and MCF7^{Notch4}) were generated, as well as a scrambled control (MCF7^{scr}). *In vitro* culture was used to assess doxycycline-induced receptor knockdown, and immunoblot analysis was carried out to measure specificity. In MCF7^{scr} cells, Notch1 and Notch4 receptor expression was not affected after addition of doxycycline. Knockdown was specific to the targeted receptor, with complete knockdown of Notch1 in MCF7^{Notch1} cells and decreased in expression of Notch4 in MCF7^{Notch4} cells (Fig. 4A). In addition, N4-ICD expression is seen to increase in MCF7^{Notch1} cells, suggesting a switch to signaling

through Notch4 when Notch1 is not available. The MCF7^{scr} cells showed no effect on MFU number (Fig. 4B) with or without doxycycline treatment. When compared with the MCF7^{scr} control cells, Notch1 knockdown (MCF7^{Notch1} plus doxycycline) resulted in a 58% decrease in MFU, whereas Notch4 knockdown (MCF7^{Notch4} plus doxycycline) caused a 75% decrease (Fig. 4B; $P < 0.05$). Notch4 knockdown caused a significantly greater decrease in MFU than Notch1 ($P < 0.05$).

Next, *in vivo* tumor-forming ability for each shRNA cell line was assessed by s.c. injection into athymic nude mice. Table 1 shows the tumor take and final tumor volume in mice injected with 3×10^6 MCF7^{scr}, MCF7^{Notch1}, and MCF7^{Notch4} cells with/without doxycycline. Tumors formed in all control mice (MCF7^{scr} plus or minus doxycycline) by day 11, in 100% of the MCF7^{Notch1} minus doxycycline mice by day 12, and in 60% of MCF7^{Notch4} minus doxycycline by day 18. No significant difference was seen between the tumor volume of these groups ($P > 0.05$).

Two of three Notch1 knockdown mice (MCF7^{Notch1} plus doxycycline) formed tumors, and tumor volume was not significantly different from control groups ($P = 0.08$). However, there was a significant difference in the tumor growth rate in the Notch1 knockdown mice (Fig. 4C; $P = 0.02$). These results were comparable with the inhibition of Notch signaling using DBZ and suggest a role for Notch1 in tumor growth and proliferation. In contrast, no tumors formed by day 28 in Notch4 knockdown mice (MCF7^{Notch4} plus doxycycline; Fig. 4D; Table 1). Taken together, these results suggest that Notch4 inhibition has a greater effect on BCSC than Notch1 inhibition and that Notch4 is required for tumor initiation.

Discussion

Our data suggest that BCSCs exist within breast cell lines and primary samples and that these cells are self-renewing, anoikis resistant, and tumor initiating. The collection of

Table 1. Effect of Notch inhibition *in vivo*

Cell type	MCF7		MDA-MB-231		MDA-MB-231_Numb		MCF7 ^{scr}		MCF7 ^{Notch1}		MCF7 ^{Notch4}	
	Vehicle control	DBZ	Vehicle control	DBZ	Vector control	Numb cDNA	-DOX	+DOX	-DOX	+DOX	-DOX	+DOX
Positive tumor growth/injection	4/4	2/4	3/6	3/6	7/7	0/7	8/8	9/9	4/4	2/3	3/5	0/5
Average days to growth	18	28	7.3	9.3	11	N/A	12	12	12	22	18	N/A
Mean tumor volume	145.4	14.9	18.7	15.1	33.7	N/A	211.04	192.98	136.39	44.95	100.6	N/A
Difference from control (P)	N/A	0.03	N/A	0.78	N/A	>0.001	N/A	0.12	0.42	0.08	0.12	0.01

NOTE: Table shows cell types injected s.c. into nude mice. Tumor growth was assessed twice weekly for 30 d. Mice positive for tumor growth/mice in group. Days to growth was calculated as average time until palpable tumor was present in each mouse. Final mean tumor volume was calculated as an average volume of mice positive for growth. ANOVA was used to calculate significance of difference.

Abbreviation: N/A, not available.

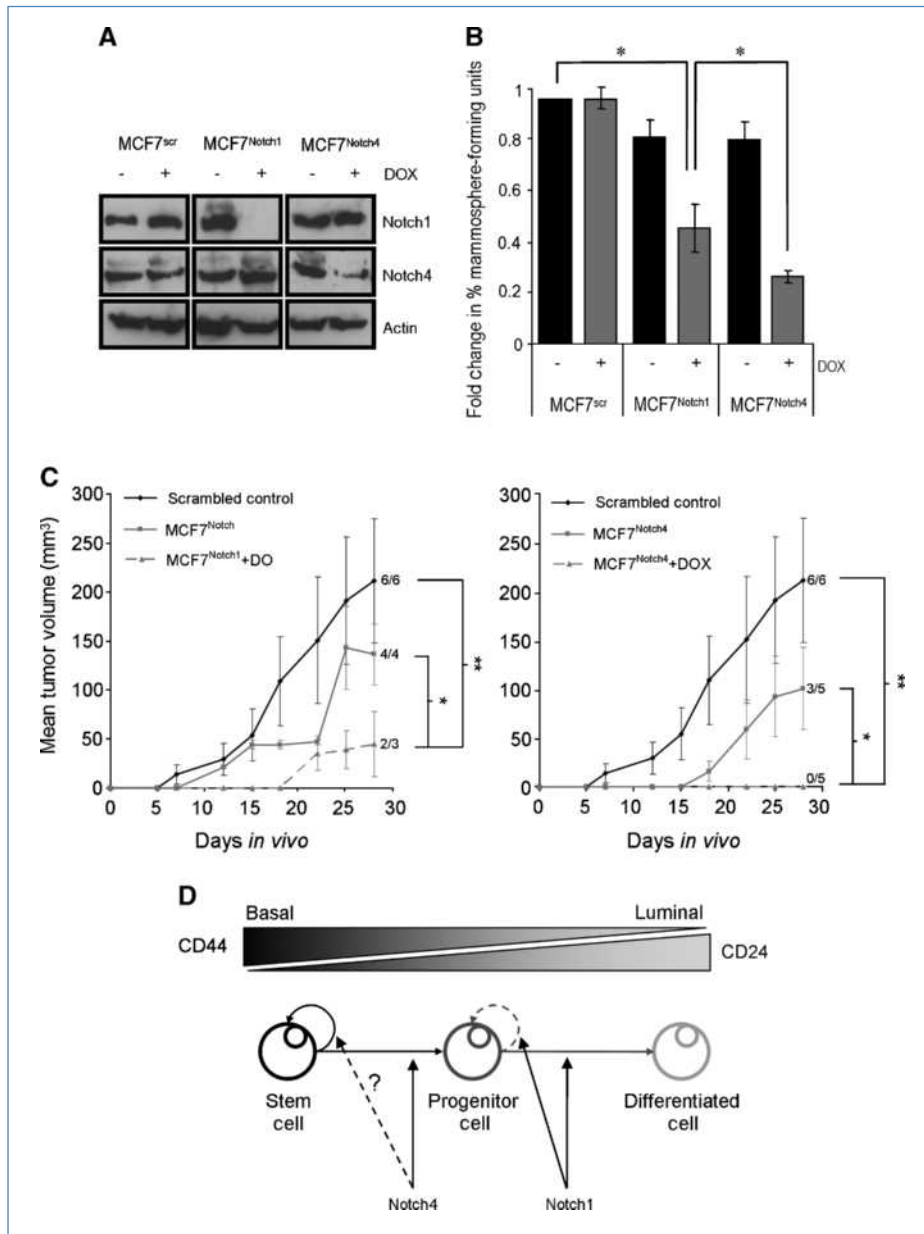


Figure 4. A, immunoblot of Notch1 and Notch4 receptors to test the extent of knockdown, and the target specificity of each shRNA cell line (actin as loading control). B, MFU number in scrambled control (MCF7^{scr}) and inducible shRNA cell lines (MCF7^{Notch1} and MCF7^{Notch4}) was measured after 7 d of culture with (gray columns) and without (black columns) doxycycline. Columns, mean; bars, SE. *, *P* < 0.05, small sample *t* test. C, mean tumor volumes over 28 d *in vivo* growth. *, *P* < 0.05; **, *P* < 0.01. D, a putative model for Notch signaling activity in the cellular hierarchy of breast cancer.

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anoikis-resistant cells isolates a BCSC-enriched subpopulation with 70% of cells expressing ESA⁺/CD44⁺/CD24^{low}. These cells are self-renewing, mammosphere, and tumor-initiating as defined by *in vitro* and *in vivo* assay techniques.

Aberrant Notch signaling has been shown to play an important role in breast cancer (16, 19). Most work to date has compared cancerous tissue with the normal breast, but we aimed to elucidate Notch receptor signaling pathway activity within BCSCs. Our results show that cell lines and primary cells have differential activity of Notch1 and Notch4 receptors in BCSCs. In the BCSC-enriched population, the majority of the Notch4 receptor is present in the cleaved/activated form. In normal human breast, Raouf and colleagues (25) showed Notch1 mRNA to be highly expressed in luminal pro-

genitor cells, whereas Notch4 mRNA was at higher levels in basal cells. In agreement with these findings, we observed more NI-ICD in the luminal cells of normal breast epithelium, whereas more nuclear Notch4 staining was detected in a basal cell population. In addition, we show that Notch4 but not Notch1 knockdown affects the percentage of ESA⁺/CD44⁺/CD24^{low} cells, suggesting that they are active in different tumor cell populations.

The differential expression of Notch1 versus Notch4 receptors in BCSCs and more differentiated cells suggests different roles for each receptor. The roles of Notch1 and Notch4 are not clearly understood in breast tissue, but Notch1 receptor has been reported to be restricted to luminal cells in the normal breast (16) and implicated in cell fate determination

(26, 27). An activating truncation of the Notch1 receptor gene is not sufficient for mammary tumorigenesis in mice. In contrast, MMTV-induced activation of the Notch4 receptor gene has been shown to inhibit mammary epithelial cell differentiation and induce tumor formation in mouse models (14). The constitutively active Notch4 results in the development of poorly differentiated basal-like tumors.

The Notch pathway has become an attractive drug target for breast cancer treatment, and the use of Notch inhibitors is predicted to be effective in reducing tumor growth, perhaps in conjunction with other treatments. GSIs, which were originally used in the treatment of Alzheimer's disease (28–30), are currently undergoing clinical trials for treatment of leukemia (31) and several solid tumors, including those of the intestine (32) and the breast (24). GSIs resensitize cancer cells to treatment with trastuzumab, tamoxifen, and chemotherapy (16, 33, 34). Our data show that inhibition of Notch signaling using both GSI and shRNA was successful in decreasing BCSC activity. However, inhibiting Notch4 receptor signaling had the greatest effect. GSI treatment inhibited Notch1 and not Notch4 activity in MCF7, BT474, and MDA-MB-231 and reduced BCSC number *in vitro* by ~50%. It has previously been suggested that Notch4 gene transcription is regulated downstream of Notch1 (35), but in the three breast cancer cell lines where we examined Notch4 activity, it was unaffected by Notch1 inhibition using GSI, suggesting Notch1-independent regulation of Notch4 (Fig. 3D). In contrast, there is a slight increase in Notch4 receptor expression where Notch1 is knocked down (Fig. 4A). This may result from differences in the percentage of ESA⁺/CD44⁺/CD24^{low} cells following DAPT compared with Notch1 knockdown shown in Fig. 3A rather than direct effects on Notch4 activity. We observed similar reductions in tumor growth *in vivo* using either DBZ or Notch1 shRNA. In comparison with GSIs or Notch1 shRNA, Notch4 shRNA had a greater inhibitory effect on mammospheres and tumor initiation. These findings suggest that a better, more specific target in BCSCs will be the Notch4 receptor. The reason why a GSI has little effect on Notch4 cleavage is unknown. Some evidence exists suggesting that a constitutively active, truncated form of the receptor exists within breast cancer cell lines (36). Another possibility is that the BCSCs are able to efflux the GSI at the dose given and they are, therefore, unaffected by treatment. The ability to efflux drugs is a known characteristic of stem cells (37), and this remains a plausible explanation.

We propose a model (Fig. 4D) where Notch4 regulates exit of BCSCs into the proliferating progenitor population, whereas Notch1 activity regulates progenitor proliferation and lu-

minal differentiation. This model may explain why DAPT and Notch1 knockdown can partially inhibit MFUs and tumor formation through their effects on progenitor proliferation. The superior effect of Notch4 knockdown on these processes would be explained by preventing the production of progenitors downstream of the stem cell-enriched population (P1). It remains unknown whether Notch4 also has a direct effect on stem cell self-renewal activity.

Overall, our findings indicate that specifically targeting the Notch4 receptor in BCSCs for treatment of breast cancer will be superior to inhibiting γ -secretase or targeting the Notch1 receptor. This is highly topical because GSIs to target Notch receptors are currently in clinical trials in combination with taxanes to which BCSCs are resistant (11). Our results indicate that Notch4 receptor plays an important role in the control of BCSC activity in cell lines and primary samples representative of different tumor types. Further work is required to assess whether this is true in all subtypes of breast cancer using primary tumor samples.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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