A Monoclonal Antibody against Human Notch1 Ligand–Binding Domain Depletes Subpopulation of Putative Breast Cancer Stem–like Cells

Ankur Sharma, Anurag N. Paranjape, Annapoorni Rangarajan, and Rajan R. Dighe

Abstract

Overexpression of Notch receptors and ligands has been associated with various cancers and developmental disorders, making Notch a potential therapeutic target. Here, we report characterization of Notch1 monoclonal antibodies (mAb) with therapeutic potential. The mAbs generated against epidermal growth factor (EGF) repeats 11 to 15 inhibited binding of Jagged1 and Delta-like4 and consequently, signaling in a dose-dependent manner, the antibodies against EGF repeats 11 to 12 being more effective than those against repeats 13 to 15. These data emphasize the role of EGF repeats 11 to 12 in ligand binding. One of the mAbs, 602.101, which specifically recognizes Notch1, inhibited ligand-dependent expression of downstream target genes of Notch such as HES-1, HES-5, and HEY-L in the breast cancer cell line MDA-MB-231. The mAb also decreased cell proliferation and induced apoptotic cell death. Furthermore, exposure to this antibody reduced CD44Hi/CD24Low subpopulation in MDA-MB-231 cells, suggesting a decrease in the cancer stem–like cell subpopulation. This was confirmed by showing that exposure to the antibody decreased the primary, secondary, and tertiary mammosphere formation efficiency of the cells. Interestingly, effect of the antibody on the putative stem-like cells appeared to be irreversible, because the mammosphere-forming efficiency could not be salvaged even after antibody removal during the secondary sphere formation. The antibody also modulated expression of genes associated with stemness and epithelial–mesenchymal transition. Thus, targeting individual Notch receptors by specific mAbs is a potential therapeutic strategy to reduce the potential breast cancer stem–like cell subpopulation.

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Introduction

Notch receptors are single-pass transmembrane receptors that are involved in normal cell growth, differentiation, and death in multicellular organisms, in a context-dependent manner (1). The N-terminal part of Notch extracellular domain (N-ECD) consists of a series of epidermal growth factor (EGF)-like repeats (ELR) that are required for ligand binding (2). Notch signaling relies on the ability of membrane-bound ligands of Delta/Serrate/Lag-2 (DSL) family to bring about conformational changes in the negative regulatory region (NRR) of the receptor, followed by a series of proteolytic events within the transmembrane region, catalyzed by ADAM/TACE metalloproteases and γ-secretase (3–6). These proteolytic events release Notch intracellular domain (N-ICD) into the nucleus, which then associates with the DNA-binding proteins to assemble a transcription complex that in turn activates the downstream signaling cascade (7).

In addition, defects in Notch signaling lead to a large number of pathologic conditions (8). Both overexpression and downregulation of Notch receptors and ligands have been implicated in human cancers (9–12). Many reports suggest a strong correlation between coexpression of Notch receptors and its ligands in the breast cancer pathogenesis, which in turn leads to poor prognosis and survival in different cohorts (13–17). Recent studies have suggested presence of cancer stem cell (CSC) subpopulation in various cancersous tissues including breast cancers (18). As in the case of normal stem cells, Notch signaling plays an important role in maintenance of CSCs. Activation of Notch signaling results in elevated self-renewal, as shown by increased mammosphere formation, whereas inhibition of Notch signaling had the opposite effect (19). Inhibition of Notch signaling also affected the tumour-sphere-forming capacity of breast cancer cells (20). Thus, Notch signaling provides a potential therapeutic target for breast cancer treatment.

Different strategies are being developed to block Notch signaling for therapeutic targeting (21–23), the most prominent being inhibition of proteolytic
cleavages by γ-secretase inhibitors (GSI; refs. 24, 25). However, GSIs being universal inhibitors of γ-secretases also inhibit other signaling pathways (26) and have limited therapeutic potential because of their serious adverse effects (27, 28). Therefore, it is essential to generate tools that can exclusively target Notch signaling pathway and specific antibodies are the ideal molecules. These antibodies could be against the ligands (29, 30) or different domains of receptor that can distinguish between individual Notch receptors. The anti-ligand antibodies are likely to affect ligand binding to all Notch receptors leading to pan-Notch inhibition and may not be useful in pathologic situations precipitated by the paralogue-specific Notch signaling. This is particularly the case for breast cancers, where overexpression of Notch1 is correlated with poor prognosis whereas that of Notch2 is associated with good clinical outcomes, thus highlighting the importance of individual receptor-specific inhibitors (14, 31).

Individual receptor-specific antibodies against Notch1 and Notch3 NRR domains have been shown to stabilize the receptors in an autoinhibited state and are allosteric in nature (32–34). However, antibodies specific for the ligand-binding domain of the receptor can serve dual functions of being the potential therapeutic tools while providing insights into the mechanism of ligand binding and subsequent receptor activation. Previous attempts of generating antibodies against human Notch1 EGF repeats 1 to 36 suggest that these antibodies can inhibit ligand-dependent Notch activation, but their precise epitopes have not been mapped and their mechanism of action needs to be investigated (32). In present study, we report production and characterization of antibodies against EGF repeats 11 to 15 of Notch1 and show that one of the monoclonal antibodies inhibits ligand binding and consequently, signaling. We further show that this antibody can inhibit cell proliferation and particularly target the putative CSCs by modulating expression of genes associated with stemness, as well as, the epithelial–mesenchymal transition (EMT) markers. This antibody also inhibited proliferation and induced apoptotic cell death in breast cancer cell lines.

Materials and Methods

Generation of stable cell lines

The stable cell lines overexpressing human Notch1 (hN1) and human Notch2 (hN2) were generated by transfecting HEK293 cells with the respective cDNAs cloned into pcDNA3.1MyC/His (Invitrogen). The Notch ligands, hjagged1, hjagged2, hDelta-like1, and hDelta-like4, were expressed as Fc fusion protein by cloning the respective cDNAs into pFUSE-Fc1-IgG1 (InvivoGen) and purified from the culture supernatant by Protein-A affinity chromatography (GE Healthcare). MCF-7 and MDA-MB-231 cells were procured from American Type Culture Collection. No further authentication was conducted.

Expression of Notch1 receptor fragments

A fragment of Notch1 extracellular domain was expressed as glutathione S-transferase (GST) fusion protein by PCR amplifying the cDNA encoding EGF repeats 11 to 15 (amino acids 412–601) and cloning into pGEX-4-T1 (GE Healthcare). The protein was purified from the soluble cell lysate using glutathione affinity chromatography (Supplementary Fig. S1A). The hN1 EGF repeats 1 to 12 was expressed as Fc fusion protein (generously provided by Prof. Radtke, EPFL, Lausanne, Switzerland) and purified using Protein-A affinity chromatography.

Polyclonal and monoclonal antibodies

Polyclonal antibodies were raised in the rabbits against EGF repeats 11 to 15 using the immunization protocols well established in the laboratory (35, 36). Mice were immunized with the same immunogen and monoclonal antibodies (mAb) were generated using protocols established for glycoprotein hormones (37, 38). Binding of mAbs to EGF repeats 11 to 15 and GST was determined using standard ELISA protocol, and the clones reacting with GST were eliminated by incubating the hybridoma medium with the soluble GST (1 mg/mL) and then determining binding to EGF repeats 11 to 15. All interesting mAb-producing clones were further subcloned and monoclonal establishment by isolotyping the mAbs using the Isotyping Kit (Sigma-Aldrich) and determining the cross-reactivity with other Notch fragments. The detail of antibody production is provided in the Supplementary Methods.

Flow cytometric assay

Cells expressing Notch were harvested using Dulbecco’s PBS (DPBS)-EDTA, resuspended in DPBS containing 2% FBS (Invitrogen; FBS/PBS), and incubated with the primary antibody for 40 minutes at room temperature, followed by washing, resuspension, and incubation in 0.1 mL of FBS/PBS containing fluorescein isothiocyanate–conjugated antirabbit or mouse secondary antibodies for 30 minutes at room temperature. The cells were washed, resuspended in DPBS, and analyzed using the Becton Dickinson FACSCanto. The median values were calculated using the Stat program of CellQuest by Becton Dickinson.

Luciferase reporter assay

To determine effect of anti-Notch1 antibodies on Notch signaling, functional assay for Notch signaling was developed. The cells expressing Notch1 receptor such as MCF-7, MDA-MB-231, or HEK293 cells overexpressing hN1 or hN2 were seeded (5 × 10⁴ cells per well) in a 24-well plate (Nunc) and transfected with 790 ng 12xCMS-Luc and 10 ng pGL3Basic or 800 ng pGL3 control along with 1 ng pRL-Tk (Promega) using Lipofectamine 2000 (Invitrogen) as per the manufacturer’s instructions. Ligands were provided by precoating the wells with purified Jagged1 or Delta-like4 Fc (1 μg per well). The transfected cells were
incubated with or without 10 µg/mL anti-Notch1 antibodies. Luciferase reporter activity was estimated after 36 hours using the Dual Luciferase Assay Kit (Promega) and a TD-20 Luminometer (Turner Design) following the manufacturer’s protocol.

C2C12 differentiation assay

The C2C12 differentiation assay was conducted as described previously (40). Briefly, C2C12 mouse myoblast cells (5 x 10^5) were cultured in Dulbecco’s Modified Eagle’s Media (DMEM) and 10% FBS and induced to differentiate by replace the original medium with the differentiating medium containing 2% horse serum which was replenished every day. At the end of 4-day incubation, cells were examined for expression of myosin heavy chain by a confocal Zeiss LSM 510 META microscope.

Cell proliferation and apoptosis assay

To investigate effect of anti-Notch1 antibodies on proliferation of cells endogenously expressing Notch1, MCF-7, and MDA-MB-231, the cells were seeded in a 96-well plate (5 x 10^3 cells per well) for 4 hours and incubated with or without antibodies for 72 hours. Cells were subsequently labeled with bromodeoxyuridine (BrdUrd) for 12 hours and its incorporation determined as per the protocol recommended by the manufacturer (Calbiochem).

Collection and processing of primary breast cancer tissue

Primary breast tumor tissue was obtained from the Kidwai Memorial Hospital, Bangalore, India, as per the ethical guidelines of the Institutional Review Board of the hospital and the Indian Institute of Science Bangalore, India. The tissue was aseptically minced and dissociated enzymatically using 1 mg/mL Collagenase (Sigma-Aldrich) and 100 U/mL hyaluronidase (Calbiochem) at 37°C for 16 hours with constant rotation. Breast organoids were separated by centrifugation, washed with PBS, and resuspended in DMEM-F12 with growth factors (10 mg/mL human EGF, 1 µg/mL hydrocortisone, 10 µg/mL insulin, 20 ng/mL basic fibroblast growth factor, 4 ng/mL heparin, and 1% B27), antibiotics and Fungizone, and incubated at 37°C for 6 hours. The organoids were further dissociated with trypsin to yield single cells which were filtered through a 70-µm BD cell strainer to remove clumps and to obtain a largely single-cell suspension which was used for later experiments.

Mammosphere assay

MCF-7, MDA-MB-231 (5 x 10^4 cells per well), and the enzymatically dissociated single-cell suspensions (2.5 x 10^4 cells per well) of the primary breast cancer tissues were seeded in a serum-free DMEM-F12 medium with growth factors in a semisolid medium containing methylcellulose as described earlier (19, 41). Effect of anti-Notch1 antibodies on sphere-forming efficiency of these cells was assessed by incubating the cells with or without antibodies (10 µg/mL) and culturing for 1 week, replenishing the antibody every fourth day. The primary mammospheres were counted manually using ImageJ software, trypsinized, and allowed to form secondary and tertiary spheres with or without antibodies. The same experiment was also carried out in presence of 5 µmol/L DAPT (Sigma) used as a positive control for Notch inhibition.

Results

Characterization of soluble Notch ligands

The biologic activities of purified soluble Notch ligands (Jagged1/Delta-like4 Fc; Supplementary Fig. S1B) were established by showing their binding to the full-length Notch1 receptor and also determining their ability to elicit a response using the cell-based assays. The HEK293 hN1 cells were suspended in DPBS (Ca^2+, Mg^2+ free), incubated with purified Jagged1 and Delta-like4 dissolved in Hanks’ balanced salt solution (HBSS) containing 1.26 mmol/L CaCl_2, and the ligand binding was determined by flow cytometry. As shown in the Supplementary Fig. S2A, there was specific ligand binding to Notch1 receptor. Furthermore, addition of 5 mmol/L EGTA to HBSS resulted in a significant decrease in ligand binding (Supplementary Fig. S2), suggesting that interactions of these ligands with Notch1 are also calcium-dependent, as in case of Delta-like1 (39). As shown in the Supplementary Fig. S3A, there was a dose-dependent increase in binding of Jagged1 and Delta-like4 to EGF repeats 11 to 15 protein immobilized on a plastic surface. Furthermore, preincubation of the ligands with EGF repeats 11 to 15 or 1 to 12 Fc proteins abolished their subsequent binding to Notch1-expressing cells (data not shown), showing the functional nature of the ligands as well as receptor fragments. This was confirmed by showing that both ligands stimulated 12xCSL Luc reporter activities in the HEK293 hN1 cells in a dose-dependent manner (Supplementary Fig. S3B). Similarly, the precoated ligands led to inhibition of C2C12 differentiation, as examined by the myosin heavy chain expression in myotubes, using anti-myosin heavy chain antibody (Supplementary Fig. S4).

Characterization of anti-Notch1 antibodies

Binding of polyclonal antibody to EGF repeats 11 to 15 showed that it is a high titer antibody with considerable binding to GST (Supplementary Fig. S5). Passing polyclonal antibodies through GST-NHS-Sepharose removed all GST-specific antibodies but retained the antibodies specific for EGF repeats 11 to 15 (processed polyclonal antibodies). Furthermore, the processed polyclonal antibodies could specifically bind to HEK293 hN1 in the flow cytometric assay (data not shown). A total of 35 mAbs that recognized EGF repeats 11 to 15, but not GST, were selected for further analysis. Binding of mAbs to EGF repeats 11 to 15 was determined by ELISA, and as shown in the Supplementary Table S1, several mAbs showed varied binding to Notch receptor fragments. Partial epitope mapping was carried out by determining binding of these mAbs to EGF repeats 1 to 12 Fc...
and EGF repeats 11 to 15 receptor fragments, followed by identification of those mAbs that showed binding to both the proteins, indicating that these antibodies recognize EGF repeats 11 to 12 epitope shared by both proteins. Several mAbs did not recognize EGF repeats 1 to 12 Fc fragment, indicating that the epitopes recognized by these antibodies reside in EGF repeats 13 to 15. Ability of all mAbs to recognize the full-length Notch1 was established by flow cytometric assay, as shown in the Supplementary Table S1.

### Inhibition of ligand-dependent Notch signaling by anti-Notch1 antibodies

Having characterized both, Notch ligands and antibodies, effect of antibodies on ligand-receptor interactions was investigated. The HEK293 hN1 cells were preincubated with polyclonal or monoclonal antibodies followed by incubation with either Jagged1 or Delta-like4 and binding of the ligand to the cells was monitored by flow cytometry. As shown in the Fig. 1, Table 1, and the Supplementary Table S1, the antibodies specific for EGF repeats 11 to 12 were more potent inhibitors of ligand binding than the antibodies specific for EGF repeats 13 to 15. Eight different mAbs were characterized for their effects on ligand-stimulated Notch signaling in HEK293 hN1 and hN2 cell lines. Ability of these antibodies to inhibit ligand-mediated receptor activation was in concordance with their effect on ligand–receptor interactions. As shown in Table 1, mAbs specific to EGF repeats 11 to 12 inhibited Jagged1- as well as Delta-like4–stimulated receptor activation. The mAb 602.101 was the most potent inhibitor of Jagged1/Delta-like4–stimulated Notch1 signaling but had no effect on the Notch2 signaling (Fig. 2A) clearly indicating the paralogue specificity of the mAb. Furthermore, this antibody showed a dose-dependent inhibition of Notch1 signaling (Fig. 2B) and proliferation (Fig. 2C), stimulated by both Jagged1 and Delta-like4. These results are in agreement with the previous studies suggesting that EGF repeats 11 to 12 is the most critical domain for ligand binding (39, 42).

### Inhibition of Notch signaling in breast cancer cell lines by anti-Notch1 antibody

Several studies suggest that Notch receptor and ligands are overexpressed in the breast cancer tissues compared with the normal breast epithelium (14, 27, 43, 44) and there is a strong correlation between high expression of Notch1 and Jagged1 and poor prognosis and survival (15). Because mAb 602.101 inhibited Jagged1 and Delta-like4 activities in the Notch1-overexpressing cells, ability of this antibody to inhibit Notch signaling in the cancer cell lines MCF-7 and MDA-MB-231 was investigated. As shown in the Fig. 3A for MCF-7 and in Fig. 3B for MDA-MB-231, the mAb 602.101 was able to inhibit Notch signaling in a dose-dependent manner. MDA-MB-231 cells were cultured in presence and absence of the antibody for 48 hours, and the endogenous transcript levels of Notch target genes, such as HES-1, HES-5, and Hey-L, were determined by quantitative real-time PCR (RT-PCR). As shown in Fig. 3C, mRNA levels of these 3 downstream genes were decreased in presence of mAb, clearly indicating that mAb 602.101 suppressed Notch signaling.

### Inhibitory effect of anti-Notch1 antibody on proliferation and putative CSC subpopulation in breast cancer cell lines

MCF-7 and MDA-MB-231 cells were incubated with mAb 602.101 or control IgG for 72 hours and BrdUrd incorporation into DNA was monitored. As shown in Fig. 3D and E, the mAb inhibited cells proliferation in a dose-dependent manner. To investigate the effect of mAb on CSC population, the cells were grown in suspension culture in presence or absence of the antibody and allowed to form mammospheres. The cells from cancer cell lines, as well as, the primary breast cancer tissues showed significant decrease in mammosphere-forming efficiency in presence of mAb (Fig. 4A and D). The mAb also inhibited secondary and tertiary sphere formation in breast cancer cell lines (Fig. 4C). Interestingly, the cells treated with mAb during the primary sphere formation stage could not form secondary spheres even after antibody removal suggesting that treatment with this mAb
Cell lines associated with stemness and EMT in breast cancer

Modulatory effect of anti-Notch1 antibody on genes

<table>
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<tr>
<th>Anti-Notch1 mAb</th>
<th>% binding inhibition Notch1 (n = 3)</th>
<th>% binding inhibition Notch2 (n = 3)</th>
<th>% signaling inhibition Notch1 (n = 3)</th>
<th>% signaling inhibition Notch2 (n = 3)</th>
<th>% proliferation inhibition (n = 3)</th>
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*aEpitope specificity of Notch mAbs: mAbs were incubated with either EGF repeats 11 to 15 or 1 to 12 adsorbed on a plastic surface and binding was determined. The mAbs that recognized both receptor fragments were considered as EGF repeats 11 to 12 specific, whereas others were identified as EGF repeats 13 to 15–specific mAbs.

*bThe HEK293 hN1 or hN2 cells were incubated with the control IgG or mAbs (10 μg/mL) for 1 hour followed by washing with DPBS and incubation with the saturating concentration of ligands (20 μg/mL) for 1 hour on ice. The cells were then incubated with anti-human Fc conjugated with fluorescein isothiocyanate antibody and binding was determined by flow cytometry. Ratio of median fluorescence intensity in the presence of control IgG and Notch1 IgG was calculated for percent inhibition.

*cHEK293 hN1 or hN2 cells transfected with 12xCSL-Luc reporter plasmid were cultured for 36 hours on plates precoated with 20 μg/mL soluble Jagged1/Delta-like4 Fc in the presence of 10 μg/mL control IgG or anti-Notch1 IgG, and the luciferase activities were determined by dual luciferase assay. The ratio of firefly luciferase to Renilla luciferase was calculated for normalization. Ratio of normalized values for the control IgG to anti-Notch1 IgG was calculated for determining percentage of inhibition.

*dHEK293 hN1, hN2, or vector-alone cells were cultured on Jagged1 Fc precoated plates in the presence of 10 μg/mL control IgG or anti-Notch1 mAb. BrdUrd incorporation was investigated by anti-BrdUrd–specific antibody in ELISA and percentage of inhibition was determined compared with control IgG.

depletes the stem-like cell subpopulation (Fig. 4B), thus preventing the recovery. This was further confirmed by the observation that there was a significant decrease in the CD44<sup>hi/hi</sup>/CD24<sup>low</sup> subpopulation of breast CSCs when the MDA-MB-231 cells were exposed to the antibody (Fig. 5A) for 72 hours. Furthermore, the sphere-forming efficiency of CD44<sup>hi/hi</sup>/CD24<sup>low</sup> was significantly inhibited even when these cells were grown in semisolid suspension culture using methylcellulose along with the anti-Notch1 mAb (Fig. 5B and Supplementary Fig. S6). Incubation of MDA-MB-231 cells with mAb also increased Annexin V–positive cells clearly indicating that mAb induced apoptotic cell death of the cancer cells (Fig. 5C and Supplementary Fig. S7).

Modulatory effect of anti-Notch1 antibody on genes associated with stemness and EMT in breast cancer cell lines

Because mAb 602.101 inhibited mammosphere formation and reduced CD44<sup>hi/hi</sup>/CD24<sup>low</sup> population in breast cancer cell lines, effect of the antibody on expression of stemness and EMT markers was next investigated. MDA-MB-231 cells were cultured in presence of mAb for 48 hours and transcript levels of stemness (Bmi-1, Nanog, Sox-2, and Oct-4), epithelial (CK14, CK18, E-cadherin), and EMT markers (Vimentin, N-cadherin, FN1, Fox-C2, Slug1, Zeb1, Zeb2, Snai1, and Twist1) were determined by quantitative RT-PCR analysis. As shown in Fig. 5D, all 4 markers for the stemness were decreased in presence of the antibody. Furthermore, the genes associated with epithelial lineage were upregulated, whereas those associated with induction of EMT were downregulated (Fig. 5E).

**Discussion**

Antibodies in general have proved to be excellent tools to map the ligand–receptor contact points and mechanism of receptor activation (38, 45). Recently, it was shown that antibodies against Notch receptor EGF repeats inhibit ligand–receptor interactions and signal transduction (32, 33). In the present study, antibody approach has been used for in-depth analysis of Notch receptor–ligand interactions and potential therapeutic applications. Several mAbs were characterized for their effects on ligand binding and consequent receptor activation. The mAbs specific for EGF repeats 11 to 12 appeared to be most effective in inhibiting Jagged1 and Delta-like4 binding to Notch1 receptor and subsequent response confirming the importance of EGF repeats 11 to 12 in the Notch ligand–receptor interaction.

**Table 1. Characterization of Notch mAbs**

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this mAb to detect conformational changes in the receptor, increased in the presence of calcium, indicating ability of mAb 602.101 binding was also decreased significantly on chelation (Supplementary Fig. S2). Interestingly, mAb 602.101 binding was also decreased equally, EGF repeats 11 to 12 could be the primary binding site for all Notch ligands, as the same domain has binding and signal transduction via a mechanism different from that of the EGF repeats 11 to 12 antibodies against other domains of Notch can modulate ligand binding cannot be completely ruled out and antibodies interactions. However, role of other EGF repeats in ligand binding to pathogenesis (15, 43, 46). Therefore, several attempts have been made to inhibit Notch function by small-molecular inhibitors such as GSIs or short interfering RNA-mediated approaches. However, general inhibitors of γ-secretase have wide ranging effect, affecting at least 20 different signaling pathways (47) and as discussed above, several adverse effects of such inhibitors have already been reported. Similarly, the short interfering RNA approach is also impractical as a general therapeutic strategy. However, domain-specific antibodies can be the magic bullets thus unraveling the receptor activation process (Supplementary Fig. S8).

Overexpression of Notch receptors and ligands and consequent increase in Notch activity has been reported in number of cancers, particularly the breast cancer and their early precursors, linking upregulated Notch signaling to pathogenesis (15, 43, 46). Therefore, several attempts have been made to inhibit Notch function by small-molecular inhibitors such as GSIs or short interfering RNA-mediated approaches. However, general inhibitors of γ-secretase have wide ranging effect, affecting at least 20 different signaling pathways (47) and as discussed above, several adverse effects of such inhibitors have already been reported. Similarly, the short interfering RNA approach is also impractical as a general therapeutic strategy. However, domain-specific antibodies can be the magic bullets
for targeting the Notch-associated pathobiology. Effectiveness of anti-NRR antibodies in targeting oncogenic Notch signaling in T-acute lymphoblastic leukemia (T-ALL) cell lines has already been reported (32, 34). Here, we show effectiveness of the EGF repeats 11 to 12 specific mAbs in selectively affecting ligand-dependent Notch function in breast cancer cell lines. The preliminary evidence also suggests that it can affect the primary breast tumor cells.

Inhibition of Notch signaling leads to reduction in mammosphere-forming capacity (19, 41, 43), hallmark of putative stem-like cell subpopulation. The mAb 602.101 inhibited cell proliferation and mammosphere formation up to 3 generations in cell lines, as well as, the primary tumor cells, suggesting a strong therapeutic prospect of this antibody. Furthermore, once treated with the antibody, the cells were unable to recover their stemness and could not repopulate even in absence of the antibody, clearly indicating the long-term deleterious effects of the antibody on the putative CSC subpopulation. Inhibition of Notch signaling alone has been shown to be not sufficient to inhibit neurosphere recovery and required combinational therapy with a chemotherapeutic agent such as temozolomide (48). Recent study showed that GSI MRK-003 treatment irreversibly affected sphere formation in the primary breast tumor cells, but the effect was reversible in the primary normal mammary epithelial cells suggesting differences in the sensitivities of the 2 cell populations (20). On the basis of these observations, it is tempting to speculate that irreversible inhibition of sphere formation caused by mAb 602.101 would be limited to the CSC

Figure 4. Effect of anti-Notch1 antibody on the putative CSC population. A, MCF-7 or MDA-MB-231 cells (5 × 10^4 cells) were cultured in semisolid medium (methylcellulose) in the presence of 10 µg/mL mAb 602.101, control IgG, dimethyl sulfoxide (DMSO), and DAPT (5 µmol/L), and the number of mammospheres formed was determined; magnification, ×10. B, MDA-MB-231 cells were first cultured in semisolid medium in presence of 10 µg/mL mAb 602.101 for 1 week followed by trypic digestion of spheres and cultured again for secondary sphere formation in the absence of mAb. C, quantitation of mammosphere-forming efficiency of MCF-7 and MDA-MB-231 cells in presence or absence of the antibody. D, breast cancer tissues from patients were enzymatically digested, and the single-cell suspension was incubated with 10 µg/mL mAb 602.101 in semisolid medium for 1 week, and mammosphere formation capacity was determined; magnification, ×10. E, quantification of mammosphere-forming efficiency of primary breast cancer cells in presence or absence of mAb.
subpopulation without any effect on the normal breast stem cells permanently. Furthermore, our mAb inhibited the chemotherapy- and radiotherapy-resistant CD44\textsuperscript{Hi}/CD24\textsuperscript{Low} subpopulation (49) and is potentially a strong therapeutic tool to reduce these treatment-resistant cells. The antibody also induced apoptotic cell death of the cancer cells and modulated expression of genes associated with stemness and EMT further highlighting therapeutic potential of this antibody in targeting angiogenesis and metastasis. It also altered the fate of breast cancer cells toward myoepithelial lineage as suggested by increased expression of CK14 in these cells. The exposure to mAb has probably initiated a differentiation program, leading to reduction in stem cell population.

Antibodies are proving to be an extremely interesting therapeutic strategy for cancer and a number of them...
are in various stages of therapeutic development. Recent demonstration of the role of Notch1 in breast cancer metastasis to the brain (50) suggests that targeting Notch as an effective therapeutic strategy and our antibody appears to be a promising candidate. As discussed above, different Notch receptors have different functions and regulate cell fates differently. Because Notch1 and Notch2 have opposing effects on breast cancer progression, an antibody specific for Notch1 is likely to be more effective as a therapeutic tool compared with the GSIs. In addition to its therapeutic importance, this antibody may prove to be a valuable tool in elucidating the molecular intricacies of Notch receptor-ligand interactions.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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# Molecular Cancer Therapeutics

A Monoclonal Antibody against Human Notch1 Ligand–Binding Domain Depletes Subpopulation of Putative Breast Cancer Stem–like Cells

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