

Notch1 is involved in migration and invasion of human breast cancer cells

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Abstract. The Notch pathway displays several functions related to tumor progression. Breast carcinomas commonly express Notch1, Notch2, Notch3 and Notch4 at variable levels and these are mainly involved in differentiation, proliferation and survival. Notch1 can also induce the invasion of breast cancer cells. However, the precise role and mechanism of Notch1 in tumor invasion remains unclear. In this report, we used small interference RNA technology to knock down the expression of Notch1, resulting in reduced migration and invasion of breast cancer cells. Meanwhile, F-actin polymerization, which is essential for cellular generation of the forces needed for motility, was also impaired in Notch1 knockdown cells. We further investigated the expression of extracellular matrix metalloproteinase inducer (EMMPRIN), matrix metalloproteinases-2 (MMP-2) and MMP-9, and found that the expression of functional EMMPRIN and MMP-2 was significantly decreased in Notch1 knockdown cells, while the expression of MMP-9 was constant. Additionally, the silencing of Notch1 expression likewise impaired cell-to-matrix and cell-to-cell adhesion. Western blotting results showed that reduction of Notch1 levels impacted the phosphorylation of PAK, phosphorylation of Akt, phosphorylation of FAK, the phosphorylation of integrin β 1, ICAM-1 and β -catenin. Collectively, these findings suggest that targeting Notch1 has important therapeutic value in breast cancer.

Introduction

The major reason of breast cancer-related deaths is the presence of metastases that are resistant to conventional therapies. Although most early stage cancers are successfully treated by established approaches, metastases that are unresponsive to available drugs continue to be fatal to patients and pose a major challenge for researchers and clinicians. A thorough understanding of the metastatic process is required to develop treatment strategies for breast cancer.

Notch genes encode proteins that can be activated through their interaction with a family of ligands (1,2). In mammals, there are four Notch receptors have four members, Notch1 to Notch4. These receptors have five ligands, Delta like 1, 3, 4 and Jagged 1 and 2. The interaction between Notch receptors and their ligands causes two-step proteolysis of the receptors. The full length protein is first cleaved to generate the non-covalently associated extracellular and transmembrane subunits in the Golgi. The final cleavage, catalyzed by γ -secretase, releases the NICD (Notch intracellular domain) which translocates into the nucleus where it forms a transcriptionally active complex with CSL (for CBF-1/RBP-J κ , Suppressor of Hairless, and Lag-1), histone acetyltransferases, and co-activators, such as mastermind-like (MAML), thus regulating the expression of target genes. Notch plays important roles in cell fate processes, such as cellular differentiation, proliferation, and survival in a variety of contexts (3-5).

In human cancer cells, Notch1 acts as either a tumor suppressor or an oncogene. Aberrant expression of the Notch pathway has been found in a variety of human cancers including cervical, colon, head and neck, renal cell carcinoma, acute myeloid and pancreatic cancer (6,7). The oncogenic role of Notch1 was first identified in human T-cell neoplasia (8). Mutations of Notch1 have been identified in more than 50% of human T-cell acute lymphoblastic leukemia (9). The down-regulation of Notch1 could be an effective approach for the inhibition of invasion and metastasis of pancreatic and prostate cancer (10). In human breast cancer cell lines, activated forms of Notch1 and Notch4 have been identified (11). Dontu *et al* have shown that Notch receptor activation regulates self-renewal of mammary stem/progenitor cells in mammosphere cultures (12). In addition, high expression of Notch has been correlated with poor outcome of breast cancer.

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In ductal carcinoma *in situ* (DCIS), high expression of NICD predicts a reduced time to recurrence five years after surgery (13). Hirose *et al* revealed a significant association of Her2-negative tumors with the expression of Notch1 and Notch3 (14). Knockdown of the Notch pathway results in sensitization of breast cancer cells to deionizing radiation, leading to cell death (14). In human breast cancer cell lines, Notch2 signaling was shown to induce apoptosis and inhibit human MDA-MB-231 xenograft growth (15). Another report has demonstrated that inhibition of Notch1 and Notch4 will be effective in suppressing breast cancer recurrence, as it is initiated by breast cancer stem cells (16).

Pharmacological manipulation of Notch signaling is becoming a new strategy for human cancer treatment. The γ -secretase inhibitor, originally developed for Alzheimer's disease, can inhibit the proteolytic processing of Notch receptors (17), and is being investigated clinically in T-cell leukemia and breast cancer. N-[N-(3,5-difluoro-phenacetyl-L-alanyl)]-S-phenylglycine t-butyl ester (DAPT) is an inhibitor of γ -secretase, which can efficiently block the presenilin- γ -secretase complex and, as a consequence, efficiently prevent activation of the Notch response. Previous studies have mainly indicated that the Notch pathway is involved in proliferation, survival and apoptosis. However, research about the function and mechanism of migration and invasion in breast cancer cells is limited.

In this study, we tested the hypothesis that Notch1 directly participates in breast cancer cell migration and invasion. By small RNA interference and treatment with DAPT to down-regulate the expression of Notch1, MDA-MB-231 cells exhibited significant impairment in a series of migration and invasion assays *in vitro*. Furthermore, our data demonstrate that several key proteins, including integrin β 1, FAK, Akt, ICAM-1, β -catenin, MMP-2, MMP-9 and EMMPRIN, were all involved in Notch1 signaling pathways which are critical for breast cancer cells migration and invasion. Thus, our studies suggest that Notch1 is required in breast cancer migration and invasion, and may be a novel target for therapeutic interventions for breast carcinoma infiltration.

Materials and methods

Cell culture and reagents. The human breast cancer cell line MDA-MB-231 was obtained from the American Type Culture Collection (Manassas, VA). The cells were cultured in a complete medium (RPMI-1640 supplement with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μ g/ml streptomycin) in a 5% CO₂ 37°C incubator. The recombinant human epidermal growth factor (EGF) was obtained from R&D Systems (Minneapolis, MN). The chemotaxis chambers and membranes were from Neuroprobe (Gaithersburg, MD). Matrigel and fibronectin (0.1%) were from Sigma (St. Louis, MO) and the Alexa Fluor 568 phalloidin was from Molecular Probes Inc. (Eugene, OR). The antibodies toward Notch1, GAPDH, MMP-2, MMP-9, EMMPRIN, phospho-integrin β 1, phospho-FAK, ICAM-1 were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The antibodies for Akt and phospho-Akt were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). The antibodies for integrin β 1 were from BD Transduction Laboratories (San Jose, CA, USA).

Notch1 siRNA plasmid transfection to MDA-MB-231 cells. Cells were plated in a 35-mm dish for 24 h before transfection in a complete medium. The transfection was performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Notch1-specific siRNA plasmid for MDA-MB-231 (sense sequence: 5'-AAG GUG UCU UCC AGA UCC UGA-3') (18) and the scrambled sequence (5'-AAA UGU GUG UAC GUC UCC UCC-3') inserted into pGPU6/GFP/Neo were from GenePharma Corp. (Shanghai, China). To establish the stable siNotch1/MDA-MB-231 cell lines, the G418-resistant cells were screened, and their expression level of Notch1 protein was monitored by Western blotting. Clones, in which the expression of Notch1 was effectively suppressed, were selected and used for the *in vitro* study.

Western blotting. Western blotting was performed as described by Zhang *et al* (19). Briefly, cells were washed twice with ice cold PBS, and then lysed with 1X SDS lysis buffer containing Tris-HCl (pH 6.8), 62.5 mM, 2% SDS, 10% glycerol for 20 min on ice. Samples were boiled for 10 min, followed by centrifugation at 10,000 rpm for 10 min, and isolation of the supernatants. Equal amounts of protein were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and electrotransferred onto nitrocellulose membranes (Immobilon-P, Millipore, Billerica, MA). The blots were then probed with appropriate dilutions of the primary antibody overnight at 4°C. Western blots were visualized using enhanced chemiluminescence reagents ECL or the mixture of nitrotetrazolium blue chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt (BCIP).

Proliferation assay. Cells were plated in 12-well plates at 3.5×10^4 per well and cultured in a complete medium. From the second day, cells were trypsinized and the total cell number was counted each day.

Chemotaxis assay. The chemotaxis assay was performed as previously described (19). The cells (7×10^5 cells/ml) suspended in the binding medium (RPMI-1640, 0.1% BSA, and 25 mM HEPES) were added to the upper chambers. Different concentrations of EGF (1, 10, 100, 1000 ng/ml) were loaded into the lower chemotaxis chamber. The 8- μ m pore size polycarbonate membrane was pretreated with 10 μ g/ml fibronectin in serum-free medium at 4°C overnight, dried in air, and inserted between the upper and lower chambers. Then, the chamber was incubated at 37°C in 5% CO₂ for 3 h. The membrane was then rinsed, fixed, and stained. The numbers of migrating cells were counted at $\times 200$ in 3 separate fields by light microscopy.

Scratch assay. Cells were plated in 35-mm dishes overnight to grow into a monolayer. Then, the plate was lined out with an even trace in the middle using a 10- μ l pipette tip. The cells were then incubated at 37°C in 5% CO₂ for the indicated times. The wounds were photographed at intervals and the distance of the wounds was measured by light microscopy.

Cellular F-actin measurement. The F-actin content was detected as previously described (20). Briefly, cells were

plated and cultured overnight in complete medium followed by further culturing in serum-free medium for 3 h. The cells were stimulated by 50 ng/ml EGF at 37°C at different time points, fixed, permeabilized, and incubated with Oregon Alexa Fluor 568 phalloidin in F-actin buffer (10 mmol/l HEPES, 20 mmol/l KH_2PO_4 , 5 mmol/l EGTA, 2 mmol/l MgCl_2 , PBS, pH 7.4) at room temperature for 2 h. The cells were washed five times. The labeled phalloidin that were bound to F-actin was extracted by using methanol at 4°C for 90 min. The fluorescence was captured at Ex/Em wavelength of 578/600 nm in each sample and normalized against the total protein content as analyzed by a BCA kit (Pierce, Rockford, IL). The relative F-actin content over different time periods (Δt) was calculated by the following equations: $\text{F-actin } \Delta t / \text{F-actin } 0 = \text{fluorescence } \Delta t / \text{fluorescence } 0$.

Adhesion assay. The adhesion assay was carried out as previously described (21). Cells (4×10^5 cells/ml) were suspended in complete medium and incubated at 37°C in 5% CO_2 incubator for 20 min. Then 1.5 ml of cell solution was promptly added to a 35-mm dish containing dried glass coverslips. The coverslips had been pretreated with 10 $\mu\text{g/ml}$ of fibronectin in serum-free medium for 2 h at 37°C and then air-dried for 30 min at room temperature. After 5, 15, 30 min of incubation, the cells were gently washed twice with cold PBS and then fixed with 4% paraformaldehyde for 20 min. The cells attached to the coverslips were counted by light microscopy at x200 magnification.

Aggregation assay. The cell aggregation assay was carried out according to previously described protocols (22,23). Briefly, cells were prepared according to the following conditions. A single cell suspension was generated using 4 mM EDTA with 1.0 mM calcium. After trypsinization, the cells were re-suspended in saline and poured into albumin-coated 35-mm plates, then incubated at 37°C for different time points (30, 60, 90 and 120 min). The single cell and cell clusters were counted by a hemocytometer. The aggregation index was calculated by the formula below, and was inversely correlated with cell aggregation. $\text{Aggregation index (AI)} = (\text{number of single cells} + \text{number of cell clusters}) / \text{total number of cells initially added}$.

Matrigel invasion assay. The invasion of cells *in vitro* was measured by the invasion of cells through Matrigel-coated Transwell inserts (Costar, Cambridge, MA) as previously described (21). Briefly, the Transwell inserts with 8- μm pore size were coated with a final concentration of 4 mg/ml of Matrigel. The cells were trypsinized and 200 μl of cell suspension (5×10^5 cells/ml) were added in triplicate wells. Binding medium (350 μl) (RPMI-1640, 0.1% BSA, and 25 mM HEPES) with 10 ng/ml of EGF was added to the lower well. At 37°C in 5% CO_2 , the cells were then incubated for 24 h, the non-invading cells were removed by wiping the upper side of the membrane, and the invading cells were fixed and stained. Cell invasion through the membranes were counted under a microscope in five random fields at x400 magnification.

Statistical analysis. Statistical analysis was carried out using Prism 3 from GraphPad Software (San Diego, CA). Data are presented as mean \pm SD. Statistical significance for compar-

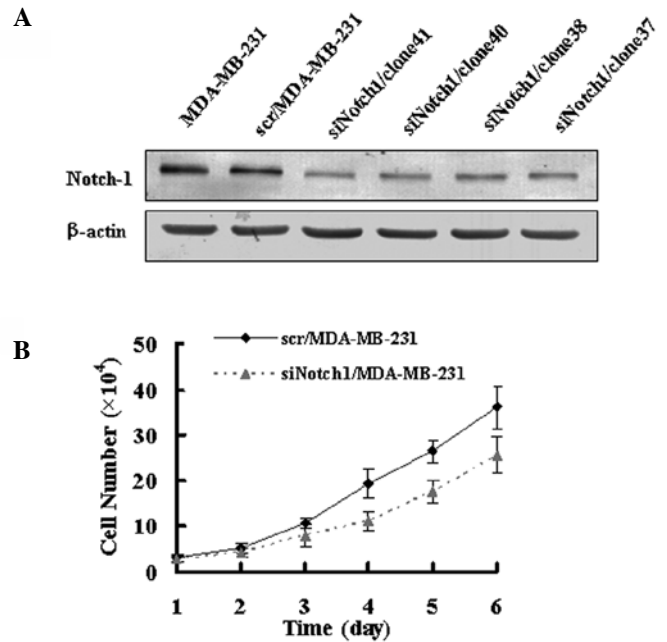


Figure 1. Establishment of stable MDA-MB-231 cells in which Notch1 was down-regulated by siRNA technology. (A) Western blot analysis of Notch1 expression in parental MDA-MB-231, scr/MDA-MB-231 and siNotch1/MDA-MB-231 cell clones. Equal amounts of protein (25 μg) were loaded on each lane. β -actin was used as a internal control. (B) Comparison of cell proliferation in scr/MDA-MB-231 and siNotch1/MDA-MB-231 cells. (A and B) Results are representative of at least three independent experiments. (B) Bars, standard deviation; two-way ANOVA analysis, $p < 0.05$.

sons between groups was determined using Student's paired two-tailed t-test. All the results were generated from three independent experiments.

Results

Notch1 expression in breast cancer cell lines. To examine the function of Notch1, we employed RNA mediated interference to suppress the expression of Notch1 in the breast cancer cell line, MDA-MB-231. Plasmid expressing Notch1 siRNA sequence was transfected into MDA-MB-231 cells to obtain stable Notch1 down-regulated cells, which were designated as siNotch1/MDA-MB-231 cells. After the G418 selection, several independent stable clones were isolated and the Notch1 expression was verified by Western blot analysis. An siRNA vector containing a scrambled sequence was also transfected to the MDA-MB-231 cells to generate control cells, which were designated as scr/MDA-MB-231 cells. The scr/MDA-MB-231 cells displayed similar Notch1 levels with the parental MDA-MB-231 cells. Notch1 protein levels were significantly reduced in different siNotch1/MDA-MB-231 clones, compared with scr/MDA-MB-231 cells, especially clone41 (Fig. 1A). In order to confirm the function of Notch1, all the subsequent functional assays and Western blot analyses were conducted using the siNotch1/MDA-MB-231 clone41 cells.

Previous studies have shown that Notch signaling is involved in cell development, including proliferation, apoptosis and tumorigenesis (3,4). Thus, we examined the effect of Notch1 silencing on the cell proliferation in MDA-MB-231 cells *in vitro*. The results of the proliferation assay indicated

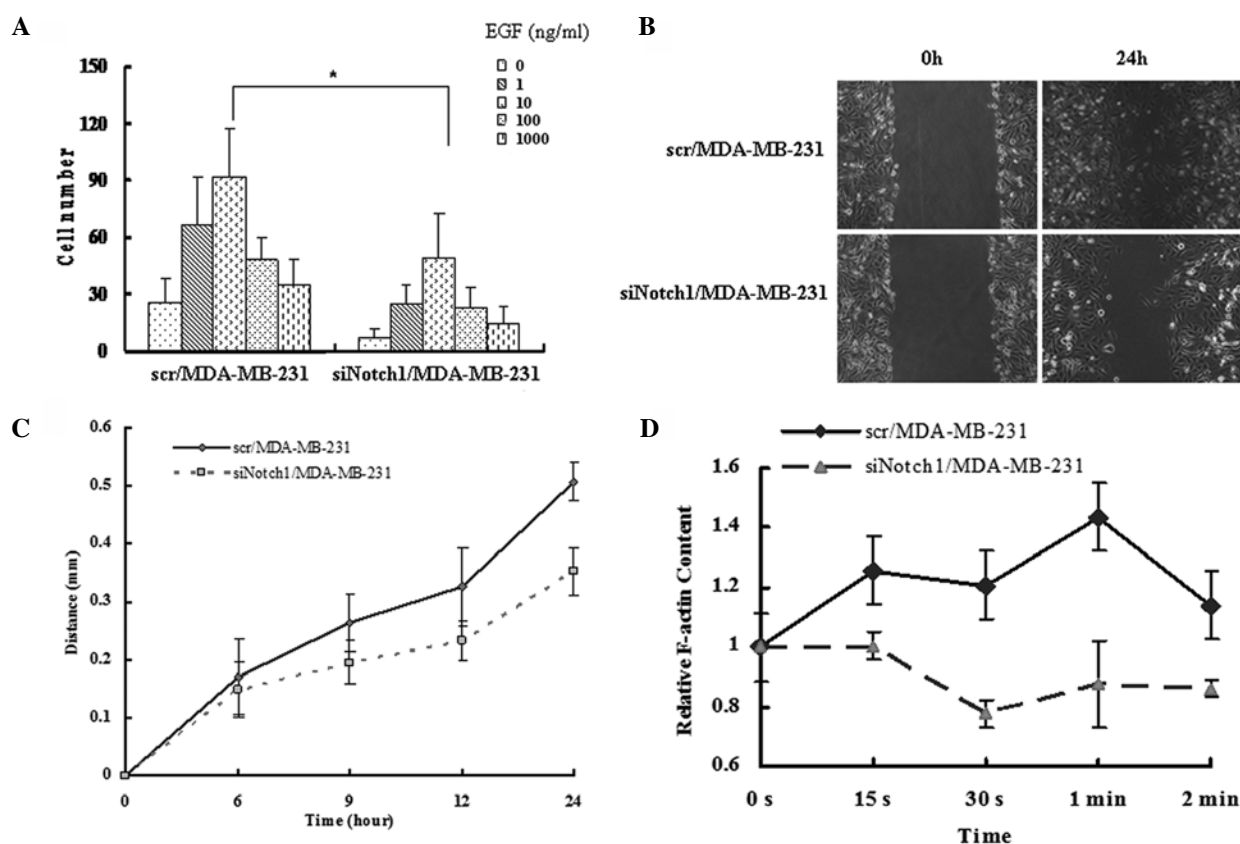


Figure 2. Reduction of Notch1 inhibited the migration of the MDA-MB-231 cells. (A) Comparison of EGF-induced chemotaxis of scr/MDA-MB-231 and siNotch1/MDA-MB-231 cells. Results are representative of three independent experiments. Columns, mean of triplicate measurements; bars, standard deviation. * $p < 0.05$, two-way ANOVA). (B) The scr/MDA-MB-231 and siNotch1/MDA-MB-231 cells were grown to confluence on a 35-mm tissue culture dish and the monolayers were wounded with a 10- μ l pipette tip. The images were photographed at 0 and 24 h (x100). (C) The migrating distance of the scr/MDA-MB-231 and siNotch1/MDA-MB-231 cells were shown at 0, 6, 9, 12, 24 h in the scratch assay (two-way ANOVA analysis, $p < 0.05$). (D) Time course of relative F-actin content in scr/MDA-MB-231 and siNotch1/MDA-MB-231 cells. Cells were stimulated by 50 ng/ml EGF for several time points before fixation and staining of F-actin by using phalloidin conjugated to Alexa Fluor 568. Data are the results of three independent experiments.

that reduction of Notch1 led to a decreased cell proliferation compared with control cells, which was consistent with a previous report (Fig. 1B).

Reduction of Notch1 impaired the migration ability of breast cancer cells. To investigate whether reduction of Notch1 affects the migration of MDA-MB-231 cells, we conducted three-dimensional cell migration assays using a 48-well chemotaxis model. As illustrated in Fig. 2A, we found that siNotch1/MDA-MB-231 clone41 cells displayed significant reduced chemotaxis ability, comparing with the control cells.

To further confirm the effects of Notch1 on MDA-MB-231 cell migration, a two-dimensional wound healing assay was also performed. After making the scratch in the fluent monolayer cells, scr/MDA-MB-231 cells migrated into the wound and resulted in wound closure within 24 h, whereas siNotch1/MDA-MB-231 clone41 cells were significantly less motile as supported by the delay in the mean distance of closure (Fig. 2B and C). Taken together, these results illustrate that Notch1 reduction by small RNA interference technology impaired the migration ability of breast cancer cells *in vitro*.

The development of membrane protrusions and the formation of lamellipodia at the leading edges are necessary for

cell movement. During this process, actin filaments (also called F-actin) regulate many aspects of dynamic cell motility (24,25). We used the F-actin polymerization assay to investigate the hypothesis that Notch1 could affect MDA-MB-231 cell migration by regulating F-actin polymerization. The results showed that EGF elicited a transient actin polymerization at 15 sec and 1 min in the two groups, however, the peaks of siNotch1/MDA-MB-231 clone41 cells were lower than that of the control cells (Fig. 2D). The data demonstrate that reduction of Notch1 significantly impaired F-actin polymerization in MDA-MB-231 cells, explaining the possible reason for the decreased migration ability.

N-[N-(3,5-difluoro-phenacetyl-L-alanyl)]-S-phenylglycine t-butyl ester (DAPT) is an inhibitor of γ -secretase, which can efficiently block the presenilin- γ -secretase complex and, as a consequence, efficiently prevent activation of the Notch response. Western blot analysis showed that the expression of NICD in MDA-MB-231 cells by treatment with 100 or 150 μ M DAPT decreased significantly compared to control cells (Fig. 3A). We further examined the cytotoxicity of DAPT on MDA-MB-231 cells by the MTT assay. Almost no cell cytotoxicity was observed after DAPT treatment (50, 100 or 150 μ M) (Fig. 3B). To further investigate the function of Notch1 on migration after treatment with DAPT, we used the scratch assay and the chemotaxis assay in MDA-MB-

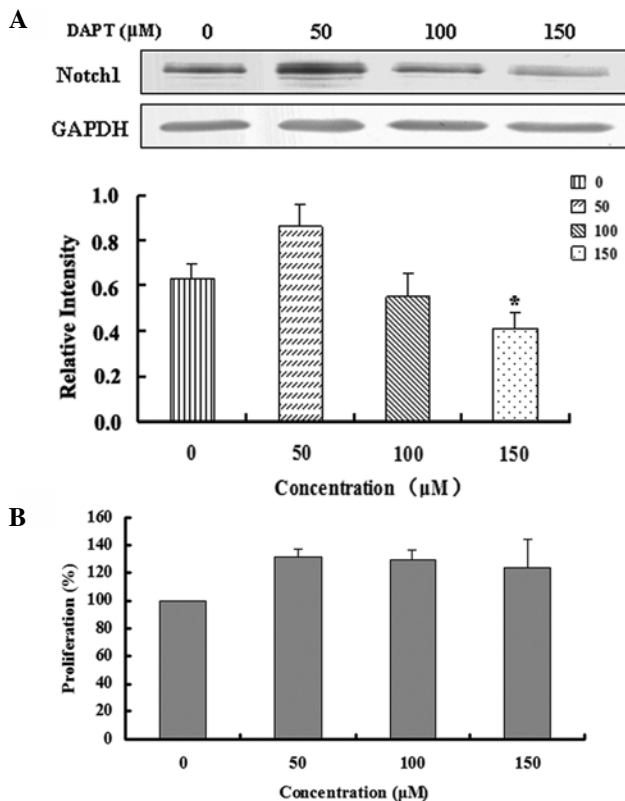


Figure 3. The expression of Notch1 is inhibited by DAPT treatment in MDA-MB-231 cells. (A) Western blot results of Notch1 in MDA-MB-231 cells which were treated with DAPT (0, 50, 100 and 150 μ M). The relative intensity of Notch1/GAPDH from triplicate samples of Western blots was calculated. Results are representative of three independent experiments. Columns, mean of triplicate measurements; bars, standard deviation; *p<0.05, two-way ANOVA. (B) Comparison of cell proliferation by the MTT assay after treatment with different concentration of DAPT (0, 50, 100 and 150 μ M) for 24 h (two-way ANOVA analysis, p>0.05).

231 cells. The migration ability of MDA-MB-231 cells was impaired by treatment with 150 μ M of DAPT (Fig. 4), which was consistent with the above results.

Reduction of Notch1 impairs the adhesion ability of breast cancer cells. Previous reports have shown that the decreased ability of cell-cell or cell-substrate adhesion is correlated with a decreased ability of cell migration (19,26,27). We have shown that reduction of Notch1 affects the migration of breast cancer cells, thus, in the next step we examined whether Notch1 affects the cell-substrate adhesion ability of MDA-MB-231 cells by an adhesion assay. The results demonstrate that the number of adherent siNotch1/MDA-MB-231 clone41 cells was significantly decreased at 15 min compared with their control cells (Fig. 5A).

It is well-known that cell adhesion molecules play an important role in breast cancer development. In this study, we used Western blot analysis to observe the changes of EGF-induced PAK, Akt, integrin- β 1 and FAK activities with Notch1 reduction in MDA-MB-231 cells. As shown in Fig. 5B, phosphorylated PAK, phosphorylated Akt, phosphorylated integrin β 1 and phosphorylated FAK were severely impaired in the siNotch1/MDA-MB-231 clone41 cells, consistent with the reduction in adhesion.

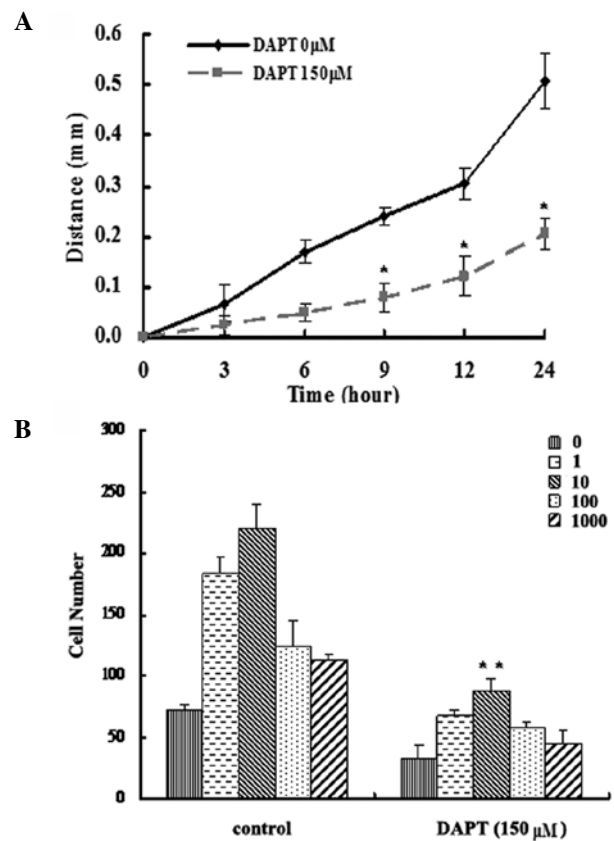


Figure 4. Inhibition of Notch1 by 150 μ M DAPT disrupts the migration of MDA-MB-231 cells. (A) After treatment with 0 and 150 μ M of DAPT for 24 h, the migrating distances of the MDA-MB-231 cells were shown at 0, 3, 6, 9, 12 and 24 h in the scratch assay (two-way ANOVA analysis, *p<0.05). (B) MDA-MB-231 cells were treated with 0 or 150 μ M of DAPT for 24 h, and the chemotaxis assay was performed in the presence of different concentrations of EGF (0, 1, 10, 100, or 1000 ng/ml). The results are representative of three independent experiments. Columns, mean of triplicate measurements; bars, standard deviation. **p<0.05, two-way ANOVA.

Disruption of intercellular adhesion by alterations of cell adhesion molecules is a key step in invasive growth (28). In this study, we also detected the cell-cell adhesion ability by an aggregation assay. The aggregation index (AI), which is inversely proportional to cell aggregation, was measured at various time points over a 2-h incubation period. As illustrated in Fig. 6A and B, the siNotch1/MDA-MB-231 cells showed a more rapid aggregation than the control cells, and the AI of siNotch1/MDA-MB-231 cells was significantly reduced after 60 min. ICAM-1 and β -catenin are two critical cell-cell adhesion proteins of cancer cells (29,30) and are associated with the development and progression of invasive tumors (31). Thus, we also assessed the expression of β -catenin and ICAM-1 and found that β -catenin and ICAM-1 expression were significantly increased in the siNotch1/MDA-MB-231 cells compared with the control cells, consisting with the enhanced aggregation assay result (Fig. 6C).

Reduction of Notch1 inhibits breast cancer cell invasion. The decrease of the migratory ability of cells usually leads to a decrease in their invasive ability. To evaluate whether Notch1 contributes to the invasion ability of MDA-MB-231 cells, we used the Matrigel invasion assay to examine the invasive potential of Notch1 siRNA-transfected MDA-MB-231 cells.

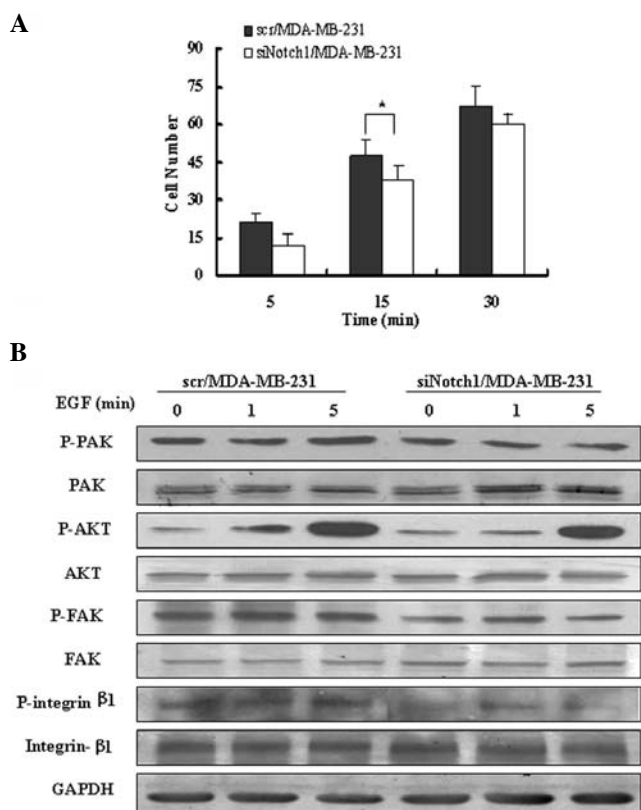


Figure 5. Disruption of Notch1 by siRNA impairs adhesion of the MDA-MB-231 cells. (A) Comparison of EGF-induced adhesion after cell treatment overnight with 0 and 150 μ M DAPT for 5, 10 and 30 min, respectively. Cell numbers were counted in five fields on every coverslip under a microscope (x200). Results are representative of three independent experiments. Columns, mean of five measurements; bars, standard deviation. (B) Western blot analysis of the phosphorylated state of PAK, AKT, FAK and integrin β 1 in total cell lysates from the control and the Notch1 impaired cells which were stimulated with 10 ng/ml EGF for 0, 1 or 5 min, respectively. PAK, AKT, FAK and integrin β 1 were used as loading controls for the respective phosphorylated proteins.

EGF (10 ng/ml) was used as a chemo-attractant to stimulate the cells to penetrate through the Matrigel and invade through the filters toward the lower chamber. The siNotch1/MDA-MB-231 clone41 cells showed a low level of penetration through the Matrigel-coated membrane compared with the control cells. The number of the invaded MDA-MB-231 cells was decreased significantly in the siNotch1/MDA-MB-231 group compared with that of the control group ($p < 0.01$; Fig. 7A and B).

In order to assess how Notch1 regulates MDA-MB-231 cell invasion, we focused on delineating the relationship between Notch1 and MMPs, particularly the MMP-2 and the MMP-9 expression, which are highly related to the progression of breast cancer. Extracellular matrix metalloproteinase inducer (EMMPRIN) has been reported to promote the release of pro-MMP-2 (32), so we detected the expression of MMP-2, MMP-9 and EMMPRIN by Western blotting. As shown in Fig. 7C, the expression of the EMMPRIN and MMP-2 were significantly decreased in the siNotch1/MDA-MB-231 clone41 cells, compared with the control, while the expression of MMP-9 was constant. These results suggest that Notch1 may be involved in MDA-MB-231 cell invasion by regulation of EMMPRIN and MMP-2.

Discussion

The Notch signaling pathway is involved in the development of the mammary gland, and also is found to be aberrantly activated in breast cancer. Notch family is related to the genotypes of breast cancer. Increased expression of Notch2 in carriers of SNP (single nucleotide polymorphism) may promote development of estrogen receptor (ER) positive luminal tumors (33). The Notch3 signaling pathway plays crucial roles in the proliferation of ErbB2-negative human breast cancer (34). In the early 1990s, Callahan and colleagues identified Notch4 as a potent oncogene, selected for mouse virus-induced mammary tumors (35,36). In fact, activated Notch4 slowed ductal growth and perturbed lobular outgrowth prior to induction of tumor formation (37). It has also been reported that the Notch1 signaling pathway could regulate the migration and metastasis of prostate cancer (38), glioblastoma (39) and pancreatic cancer (10). Chen *et al* have reported that inhibition of the Notch1 pathway abrogated the hypoxia-mediated increase in Slug and Snail expression, as well as decreased breast cancer cell migration and invasion (40). However, the mechanistic role of Notch1 signaling and the consequence of its down-regulation in breast cancer have not been fully elucidated. In this study, we reduced the expression of Notch1 by small interfering RNA (siRNA) and DAPT to further investigate the possible molecular mechanisms involved in the migration and invasion in human breast cells.

Cell migration in response to chemotactic stimuli is a key aspect of many physiological and pathological processes (41). Chemotaxis plays a central role in various biological processes, such as cellular morphogenesis, innate immunity, inflammation and metastasis of cancer cells (42-45). We used the wound healing assay and EGF-induced chemotaxis assay to investigate whether Notch1 has a function on the migration of MDA-MB-231 cells. The results showed that Notch1 reduction by siRNA attenuated the cell migration ability. Meanwhile, EGF-induced chemotaxis in DAPT treated cells was also detected, and the chemotaxis ability of the cells was significantly attenuated with Notch1 reduction, consistent with the data of siRNA-mediated Notch1 reduction cells. These results support the general involvement of Notch1 in breast cancer cell migration.

Actin is one of the major ingredients of microfilaments making up the cytoskeleton, which in turn is a basic factor for cellular motility by dynamic reorganization. The process of F-actin (also called actin filaments) polymerization is quick and transient (46). The ligand-induced transient F-actin assembly correlates with the cellular chemotactic capacity (47). In the present study, we observed that Notch1 silencing significantly impaired F-actin polymerization in MDA-MB-231 cells, thus offering a possible explanation for the observed decrease in the migration ability of the cells.

The ability of cells for adhesion also plays a critical role in wide array of physiological processes including hemostasis, immune-response, inflammation, and migration (48-50). FAK (focal adhesion kinase) is tightly linked to embryonic development, tumorigenesis and adhesion. FAK can bind to the cytoplasmic tail of integrin β 1, one of the most important groups of adhesion molecules, resulting in unfolding of FAK and promotion of cell attachment (51,52). The adhesion

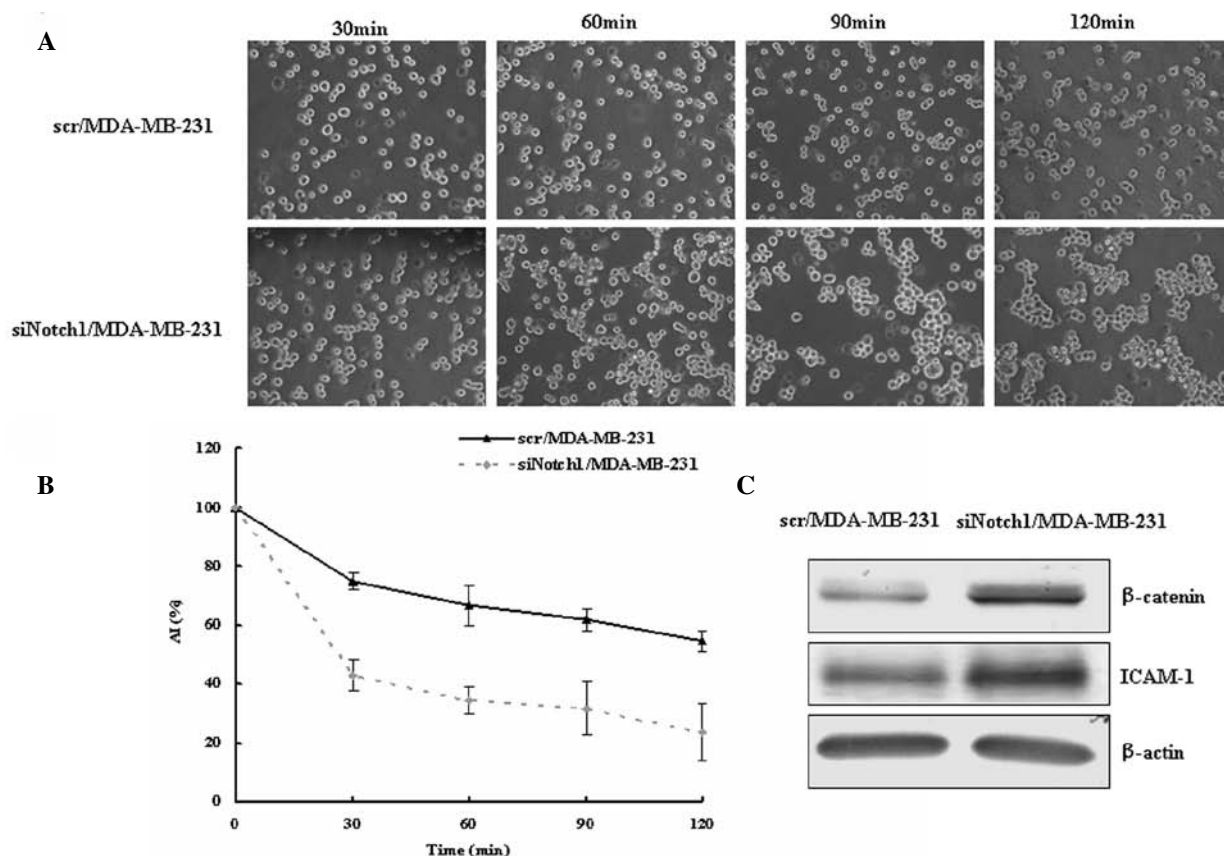


Figure 6. Disruption of Notch1 by siRNA impairs cell-substrate adhesion and cell-cell adhesion in MDA-MB-231 cells. (A) The scr/MDA-MB-231 and siNotch1/MDA-MB-231 were resuspended in saline and poured into fibronectin-coated 35-mm plates, then incubated at 37°C for different time points (30, 60, 90 and 120 min) (x100). (B) The aggregation index (AI) of the scr/MDA-MB-231 and siNotch1/MDA-MB-231 cells are shown at 30, 60, 90 and 120 min (two-way ANOVA analysis, $p < 0.05$). (C) Western blotting of β -catenin and ICAM-1 in scr/MDA-MB-231 and siNotch1/MDA-MB-231 cells. β -actin was used as a loading control. Each result is representative of at least three independent experiments.

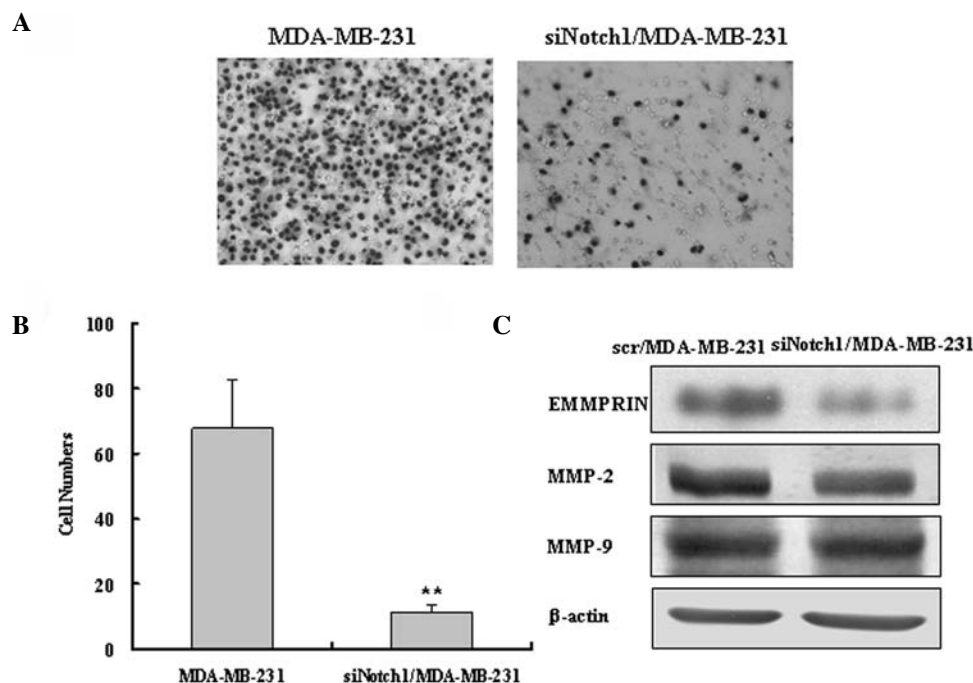


Figure 7. Disruption of Notch1 by the siRNA impairs MDA-MB-231 cell invasion *in vitro*. (A) EGF (10 ng/ml) was used as chemo-attractant, and the MDA-MB-231 and siNotch1/MDA-MB-231 cells invading through Matrigel-coated 8- μ m pore size Transwell inserts were stained as described in Materials and methods. Photographs were taken at a magnification of x200. (B) The number of invading cells was quantified by counting stained cells in five random fields of the membrane. All experiments were performed three times independently (Student's t-test, ** $p < 0.01$). (C) Comparison of the expression of EMMPRIN, MMP-2 and MMP-9 in MDA-MB-231 and siNotch1/MDA-MB-231 cells by Western blotting. β -actin was used as a loading control.

assay in our study showed that the adhesion ability of the MDA-MB-231 cells was greatly impaired by reduction of Notch1, and that EGF-induced phosphorylation of integrin $\beta 1$ was blocked in Notch1 down-regulated breast cancer cells while expression of integrin $\beta 1$ was not altered. In addition to the fact that FAK can activate the PI3K/Akt pathway (53), our study showed that the phosphorylation of Akt was decreased by the alteration of phosphorylated FAK. A previous report has suggested that Akt phosphorylated serine 21 of PAK1 resulting in a modulation of cell migration (54). We thus, further investigated the expression of PAK, and obtained a similar result. Therefore, we speculate that the inhibition of Notch1 expression blocks activation of FAK, integrin $\beta 1$, Akt and PAK, and then finally leads to the impairment of adhesion and migration.

It has been reported that cadherin-associated β -catenin promotes actin reorganization and cell-cell adhesion. ICAM-1 is another important adhesive molecule which is constitutively expressed on the cell surface (30), and is involved in cell-cell adhesion. Defects in their expression or function have been linked to tumor progression and metastasis (55). In our aggregation assay, β -catenin and ICAM-1 expression were severely increased in siNotch1/MDA-MB-231 cells consistent with their enhanced aggregation assay results, suggesting that Notch1 regulates cell-cell adhesion possibly through β -catenin and ICAM-1.

Acquisition of the invasive potential through proteinase expression is an essential event in tumor progression. MMPs (matrix metalloproteinases) are a family of proteins regulating cell invasion processes through their proteolytic function in normal and pathological states. The level of active MMPs, especially of MMP-2, is also considered to be a breast cancer metastasis indicator (56). The expression of MMP-2 is dependent on EMMPRIN, which has been implicated in several aspects of tumor progression (57). We demonstrated a significant decrease of EMMPRIN and MMP-2 expression in siNotch1/MDA-MB-231 cells, while MMP-9 expression was constant. This suggests that the Notch1 effects on the invasion of MDA-MB-231 cells is possibly mediated by MMP-2.

In summary, we have presented experimental evidence that strongly supports the antitumor effects of the down-regulation of Notch1 in MDA-MB-231 cells. Specifically, we have shown that down-regulation of Notch1 could result in the inhibition of invasion and metastasis. Thus, targeting Notch1 signaling may be of preventive and therapeutic value in breast cancer.

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