NOTCH4 Is a Potential Therapeutic Target for Triple-negative Breast Cancer

IORI NAGAMATSU[1], HIDEYA ONISHI[1], SHOJIRO MATSUSHITA[1], MAKOTO KUBO[2], MASAYA KAI[1], AKIRA IMAIZUMI[3], KENJI NAKANO[4], MASAMI HATTORI[5], YOSHINAO ODA[5], MASAO TANAKA[2] and MITSUO KATANO[1]

Departments of [1]Cancer Therapy and Research, [2]Surgery and Oncology, and [5]Anatomic Pathology, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan; [3]Shukoukai Inc., Tokyo, Japan; [4]Innovation Center for Medical Redox Navigation, Kyushu University, Fukuoka, Japan

Abstract. *Background/Aim: The prognosis for triple-negative breast cancer (TNBC) is poor. In the present study, we evaluated whether NOTCH4 receptor is a potential new therapeutic target for TNBC. Materials and Methods: In vitro proliferation and invasiveness were evaluated in TNBC cells with or without small-interfering RNA (siRNA) for NOTCH4, and with or without NOTCH4 plasmid transfection. In vivo, MDA-MB-231 cells with or without NOTCH4 siRNA were subcutaneously implanted into the flank regions of mice. The frequency of nuclear translocation of NOTCH4 was assessed by immunohistochemistry in 21 TNBC samples and 46 non-TNBC samples. Results: NOTCH4 inhibition in TNBC cells reduced proliferation and invasiveness, and NOTCH4 overexpression in TNBC cells increased proliferation and invasiveness. NOTCH4 inhibition reduced tumour volume and tumourigenicity of mouse xenografts. TNBC cells had a higher frequency of nuclear translocation of NOTCH4 than other cells. Conclusion: NOTCH4 is a new potential therapeutic target for triple-negative breast cancer.*

Breast cancer continues to be a leading cause of cancer-related death among women worldwide (1), even though it is a cancer for which many standardized adjuvant therapies are available. Of the different types of breast cancer, triple-negative breast cancer (TNBC) accounts for 15% of all breast cancers and has the worst prognosis compared to other breast cancer subtypes: luminal-A, luminal-B, estrogen receptor (ER)-negative/progesterone receptor (PR)-negative/human epidermal growth factor receptor-2 (HER2)-positive and normal-like subtypes (2). One reason for the poor prognosis of TNBC may be the lack of therapeutic targets such as ER, PR and HER2 when chemoresistance arises, although the chemotherapy response rate is relatively high. Consequently, development of an effective therapeutic strategy for TNBC is urgently required.

Morphogenesis signaling is thought to have potential for the identification of therapeutic targets for cancer. Previously, we demonstrated that the hedgehog signaling pathway, which is a morphogenesis signaling pathway, is activated in various types of cancer (3, 4), and that it contributes to the proliferation, invasiveness, and progression of cancer (5-8). In addition, pharmacological manipulation of NOTCH signaling, another morphogenesis signaling pathway, might be a new strategy for human cancer treatment (9). For example, a phase I clinical trial using the γ-secretase inhibitor, MK-0752, which inhibits NOTCH signalling, has been started for adult patients with advanced solid tumours (10). We also reported that a γ-secretase inhibitor, N-[N-(3, 5-difluorophenacetyl-L-alanyl)]-*S*-phenylglycine-t-butyl ester, has a synergic anti-tumour effect with paclitaxel or docetaxel (11). NOTCH signaling is mediated by five ligands: DELTA-like 1, DELTA-like 3, DELTA-like 4, JAGGED-1, and JAGGED-2, and -4 receptors: NOTCH-1 to -4 (12-14); and cell-to-cell contact is essential for the activation of NOTCH signalling (15). Many studies have reported the roles of NOTCH1 and NOTCH3 in breast cancer. NOTCH1 contributes to the migration and invasion of breast cancer (9), and high expression of NOTCH1 and its ligand confers a poor prognosis (16-18). Others demonstrated that HER2-negative breast cancer is correlated with the expression of both NOTCH1 and NOTCH3 (19), and that overexpression of activated NOTCH1 and NOTCH3 blocks mammary development but induces tumourigenesis of the breast (20). Only a few reports have been published

Correspondence to: Hideya Onishi, Cancer Therapy and Research, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan. Tel: +81 926426220, Fax: +81 926426221, e-mail: ohnishi@surg1.med.kyushu-u.ac.jp

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regarding the role of NOTCH4 in breast cancer, but it is known that the activated forms of NOTCH1 and NOTCH4 are present in human breast cancer (21), and that NOTCH4 receptor signalling is involved in the regulation of breast cancer stem cell activity (22). However, the function of NOTCH4 in breast cancer remains poorly-understood.

In the present study, we analyzed the biological significance of the NOTCH4 receptor in TNBC to evaluate whether the NOTCH4 receptor is a potential therapeutic target for TNBC.

Materials and Methods

Cell lines. TNBC cell lines (MDA-MB-231 and Hs578T) and non-TNBC cell lines (MCF-7 and BT-474) were obtained from the American Type Culture Collection (Manassas, VA, USA). MDA-MB-231, MCF-7, and BT-474 cells were maintained in RPMI-1640 (Nacalai Tesque, Kyoto, Japan) supplemented with 10% foetal bovine serum (FBS; Biological Industries, Kibbutz Beit Haemek, Israel) at 37˚C in 5% CO_2. Hs578T cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Nacalai Tesque) supplemented with 10% FBS at 37˚C in 5% CO_2.

RNA interference. SiRNAs for *NOTCH1* (ON-TARGETplus SMART pool L-007771), *NOTCH2* (ON-TARGETplus SMART pool L-012235), *NOTCH3* (ON-TARGETplus SMART pool L-011093), *NOTCH4* #1 (ON-TARGETplus SMART pool L-111108), and negative control siRNA were obtained from Dharmacon RNA Technologies (Chicago, IL, USA). The siRNA for *NOTCH4* #2 (sc-40137) was obtained from Santa Cruz Biotechnology (Dallas, TX, USA). Cells were transfected with 25 nM siRNA using the Lipofectamine RNAiMAX Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. At 48 h after transfection, the cells were harvested and subjected to further analyses.

Plasmids and cell transfection. The p*NOTCH4* plasmid was obtained from Promega (Madison, WI, USA) and transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. At 24 h after transfection, the cells were harvested and subjected to further analyses.

Conventional and real-time reverse transcription polymerase chain reaction (RT-PCR). Total RNA was isolated using the High Pure Isolation Kit (Roche Diagnostics Gmbh, Mannheim, Germany) and reverse-transcribed using the Quantitect Reverse Transcription Kit (Qiagen, Hilden, Germany), according to the manufacturers' protocols. Conventional and real-time RT-PCR reactions were performed using the sets of primers shown in Table I with Master Mix Kit (Qiagen) or iQSYBER Green Supermix (Bio-Rad Laboratories, Philadelphia, PA, USA), respectively. The mRNA expression of *matrix metalloproteinase (MMP) 2* and *MMP9* were also investigated by real-time RT-PCR.

Immunohistochemistry. Twenty-one TNBC samples, 46 non-TNBC samples, and 22 normal tissue samples from the margin of the tumour sections were obtained from patients with breast cancer who underwent surgery at the Kyushu University from 2004 to 2010. Approval for the use of tissues was obtained from the patients in accordance with the Ethical Committees of Clinical Study at Kyushu University (24-222). Paraffin sections of breast cancer tissues were deparaffinized and rehydrated according to routine procedures. The sections were immersed in 3% H_2O_2 and 10% goat serum and incubated with primary antibody against NOTCH4 (sc-5594, 1:100; Santa Cruz Biotechnology, CA, USA) at 4˚C overnight. The samples were incubated with HISTFINE simple stain MAX-PO(R) (Nichirei, Tokyo, Japan) and visualized using 3,3'-diaminobenzidine with haematoxylin counterstaining.

Western blot analysis. Protein samples were extracted using M-PER (Thermo Fisher Scientific, Chicago, IL, USA), lysed in sodium dodecyl sulphate (SDS) buffer, separated by SDS-polyacrylamide gel electrophoresis(PAGE), and transferred to PVDF membranes. The membranes were incubated with primary antibodies against NOTCH4 (sc-8645, 1:200; Santa Cruz Biotechnology) or α-tubulin (1:1000; Sigma-Aldrich, St. Louis, MO, USA) at 4˚C overnight, followed by peroxidase-labelled secondary antibodies at 37˚C for 1 h. Immunoblots were developed using the ECL Prime Western Blotting Detection System (GE Healthcare Life Sciences, Buckinghamshire, UK).

Proliferation assay. Cells (5×10^3 cells/well) were seeded in 96-well plates in complete culture medium. After 24, 48, and 72 h, cell growth was determined using Cell Count Reagent SF (Nacalai Tesque).

Soft agar colony-formation assay. Cells were mixed with RPMI-1640 containing 0.3% agar and 10% FBS and plated on 0.3% basal agar in six-well plates. Each well was then further covered with RPMI-1640 containing 10% FBS. Three weeks later, the colonies were stained with crystal violet (Sigma-Aldrich).

Cell invasion assay. The invasiveness of breast cancer cells was assessed based on the invasion of cells through Matrigel-coated transwell inserts as previously described (8). In brief, the upper surface of a filter (pore size, 8.0 μm; BD Biosciences, Heidelberg, Germany) was coated with basement membrane Matrigel (BD Biosciences). Cells were suspended in RPMI-1640 then 1.0×10^5 cells were added to the upper chamber and incubated for 4 h. After incubation, the filter was fixed and stained with Diff-Quick reagent (International Reagents, Kobe, Japan). All cells that had migrated from the upper to the lower side of the filter were measured using a BIOREVO BZ-9000 microscope (Keyence, Osaka, Japan). Tumour cell invasiveness testing was carried out in triplicate wells.

In vivo xenograft tumour model. Five-week-old female severe combined immunodeficiency (SCID) mice were purchased from Charles River Laboratories Japan (Kanagawa, Japan) and acclimatized for two weeks. All animal procedures were approved by the Animal Care and Use Committee at Kyushu University (A25-027-0). MDA-MB-231 cells transfected with *NOTCH4*-targeting siRNA or negative control siRNA were subcutaneously implanted into the flank regions of the SCID mice [5×10^5 and 1×10^6 cells in phosphate buffered saline (PBS) per mouse]. The tumour size was determined once a week, and the tumour volume was calculated using the following formula: length × $(\text{width})^2 \times 0.5$ mm^3.

Statistical analysis. All data are presented as the mean±SD. χ^2 test was used to analyse the tumourigenicity in mice. Student's *t*-test was used for the comparison of mean values between two groups. A value of $p<0.05$ was considered significant.

Table I. *Reverse transcription polymerase chain reaction (RT-PCR) primer sequences used in this study.*

Primers for RT-PCR		
Target	Forward primers	Reverse primers
NOTCHI	5'-GCCGCCTTTGTGCTTCTGTTC-3'	5'-CCGGTGGTCTCTCTGGTCGTC-3'
NOTCH2	5'-CACTGAGCCAAGGCATAGAC-3'	5'-ATCTGGAAGACACCTTGGGC-3'
NOTCH3	5'-TCTTGCTGCTGGTCATTCTC-3'	5'-TGCCTCATCCTCTTCAGTTG-3'
NOTCH4	5'-CACTGGGTCGATGATGAAGG-3'	5'-ATCTCCACCTCACACCACTG-3'
GAPDH	5'-CCGTCTAGAAAAACCTGCC-3'	5'-GCCAAATTCGTTGTCATACC-3'
Primers for real-time RT-PCR		
Target	Forward primers	Reverse primers
NOTCHI	5'-CCGCAGTTGTGCTCCTGAA-3'	5'-ACCTTGGCGGTCTCGTAGCT3'
NOTCH2	5'-CACCATGTACCAGATTCCAG-3'	5'-GCATAACTGTGCTGTGAAGG-3'
NOTCH3	5'-TCTCAGACTGGTCCGAATCCAC-3'	5'-CCAAGATCTAAGAACTGACGAGCG-3'
NOTCH4	5'-GCGGAGGCAGGGTCTCAACGGATG-3'	5'-AGGAGGCGGGATCGGAATGT-3'
MMP2	5'-TGATCTTGACCAGAATACCATCGA-3'	5'-GGCTTGCGAGGGAAGAAGTT-3'
MMP9	5'-TGGGCTACGTGACCTATGACAT-3'	5'-GCCCAGCCCACCTCCACTCCTC-3'
β-Actin	5'-CCAGGCACCAGGGCGTGATG-3'	5'-CGGCCAGCCAGGTCCAGACG-3'

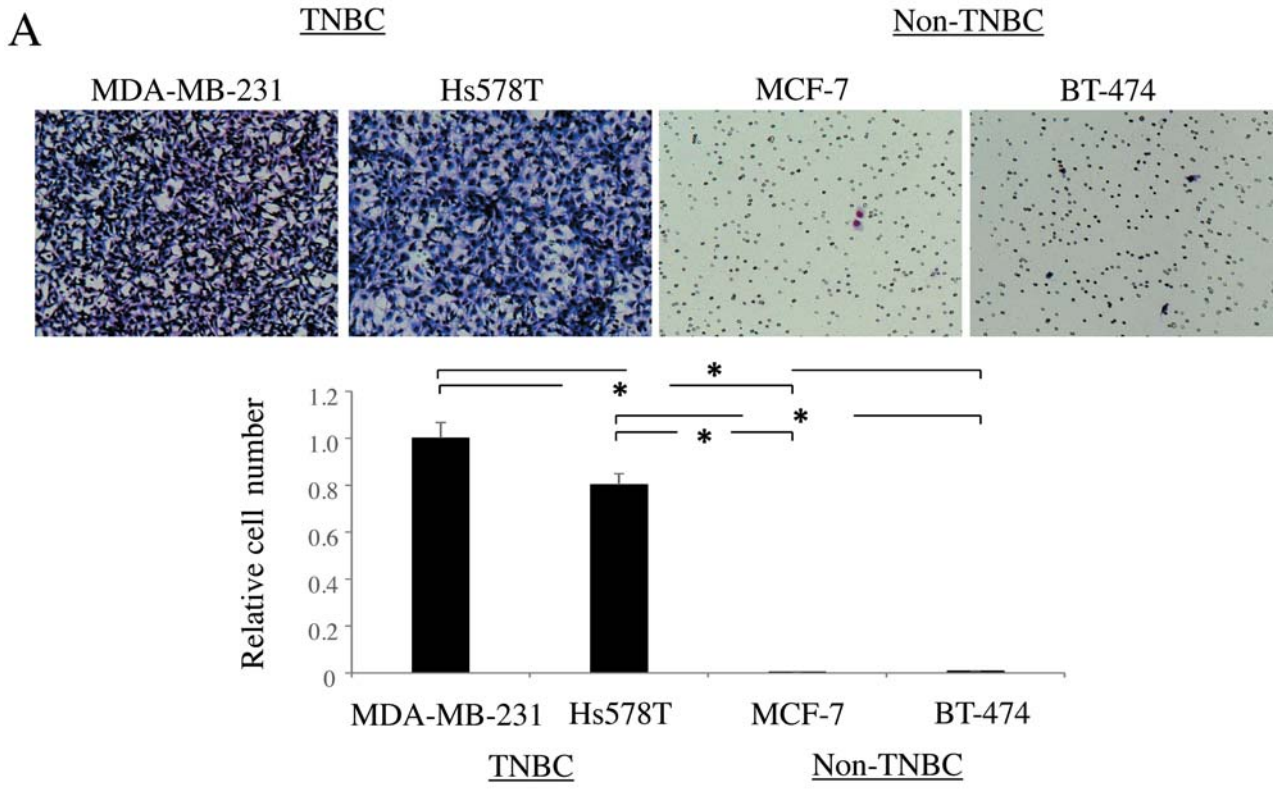
GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; MMP, matrix metalloproteinase.

Results

TNBC cells exhibited greater invasiveness and NOTCH4 expression compared to non-TNBC cells. Firstly, we compared the invasiveness of TNBC cells with that of non-TNBC cells to develop a specific therapeutic strategy for TNBC. Invasiveness of TNBC cells was significantly greater than that of non-TNBC cells (Figure 1A). Next, we investigated the expression of NOTCH1–4. Interestingly, *NOTCH4* mRNA expression in TNBC cells (MDA-MB-231 and Hs578T cells) was higher than in non-TNBC cells [MCF-7 (ER^+, $HER2^-$) and BT-474 (ER^+, $HER2^+$) cells] (Figure 1B). Therefore, we focused on NOTCH4 and investigated whether NOTCH4 contributes to the development of a malignant phenotype in TNBC cells.

Inhibition of NOTCH4 reduced proliferation, colony formation and invasiveness of TNBC cells. *NOTCH4* mRNA expression in two TNBC cell lines, MDA-MB-231 and Hs578T, was suppressed by two types of *NOTCH4* siRNA. The efficiency of *NOTCH4* mRNA-knockdown was almost 80% in MDA-MB-231 cells and about 95% in Hs578T cells (Figure 2A and B). The expression of *NOTCH1*, *NOTCH2*, and *NOTCH3* mRNAs was not affected by *NOTCH4* siRNA transfection (Figure 2A and B). Knockdown of NOTCH4 protein expression was also confirmed by western blotting (Figure 2C). Anchorage-dependent proliferation of *NOTCH4* siRNA-transfected TNBC cells was significantly lower than that of control cells (Figure 3A). In contrast, inhibition of *NOTCH1* or *NOTCH3* did not affect anchorage-dependent proliferation, while inhibition of *NOTCH2* increased anchorage-dependent proliferation, in MDA-MB-231 cells (Figure 3B). Next, anchorage-independent proliferation, which reflects a more aggressive malignant potential, was investigated using colony-formation assays. The number of colonies formed by *NOTCH4* siRNA-transfected TNBC cells was significantly lower than in control cells (Figure 3C). However, inhibition of *NOTCH1* or *NOTCH3* did not affect anchorage-independent proliferation, while inhibition of *NOTCH2* increased anchorage-dependent proliferation, in MDA-MB-231 cells (Figure 3D). Invasiveness of *NOTCH4* siRNA-transfected TNBC cells was significantly lower than in control cells (Figure 3E). In contrast, suppression of *NOTCH1* and *NOTCH3* did not affect the invasiveness, while inhibition of *NOTCH2* significantly increased invasiveness, in MDA-MB231 cells (Figure 3F). These results suggest that *NOTCH4* inhibition leads to lower proliferation, colony formation, and invasiveness of TNBC cells.

Overexpression of NOTCH4 increases proliferation, colony formation, and invasiveness in TNBC cells. To confirm whether NOTCH4 contributes to the development of a malignant phenotype in TNBC cells, *NOTCH4* was overexpressed. *NOTCH4* mRNA expression in *NOTCH4* plasmid-transfected TNBC cells was higher than in control MDA-MB-231 cells and Hs578T cells (Figure 4A). Importantly, the *NOTCH4* plasmid did not affect the expression of *NOTCH1*, *NOTCH2*, and *NOTCH3* (Figure



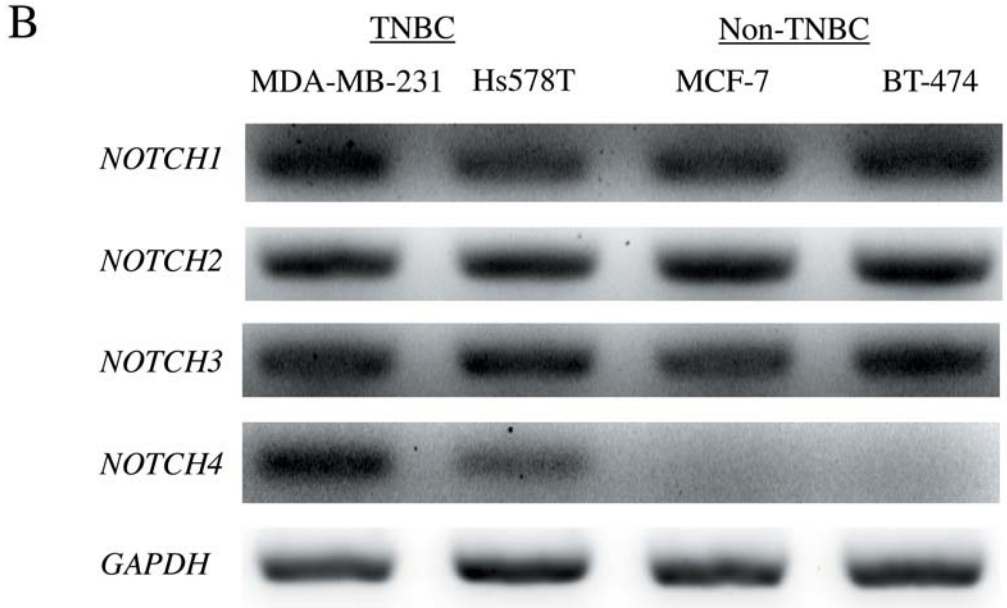


Figure 1. *Triple-negative breast cancer (TNBC) cells exhibit higher invasiveness and NOTCH4 expression compared to non-TNBC cells. A: Invasiveness of TNBC cells (MDA-MB-231 and Hs578T cells) and non-TNBC cells (MCF-7 and BT-474 cells) was investigated using Matrigel invasion assays. Representative images are shown. The original magnification was ×100. The graph shows the mean number of migrating cells±SD; *p<0.05. B: Expression of NOTCH1, -2, -3 and -4 mRNAs in TNBC cells and non-TNBC cells was estimated by conventional reverse transcription polymerase chain reaction (RT-PCR). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the loading control.*

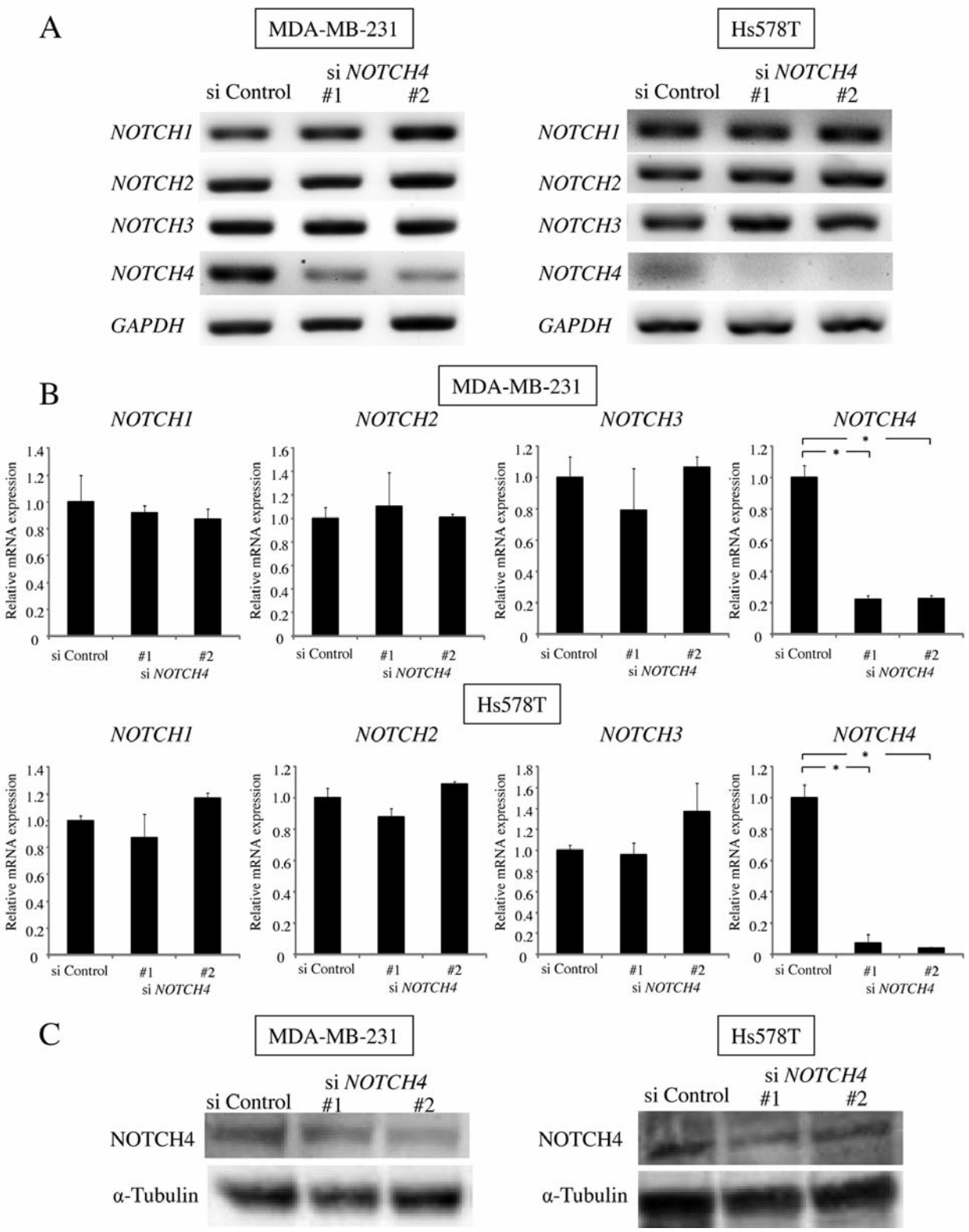


Figure 2. *NOTCH4 siRNA selectively reduces expression of NOTCH4 receptor but not those of NOTCH1,-2 and -3 receptors. A, B: Expression of NOTCH1,-2,-3 and -4 mRNAs in MDA-MB-231 and Hs578T cells was estimated by conventional and real-time reverse transcription polymerase chain reaction (RT-PCR). The graphs show the mean values±SD; *p<0.05. C: Expression of NOTCH4 protein in MDA-MB-231 and Hs578T cells was investigated by western blot analysis. α-Tubulin was used as the loading control.*

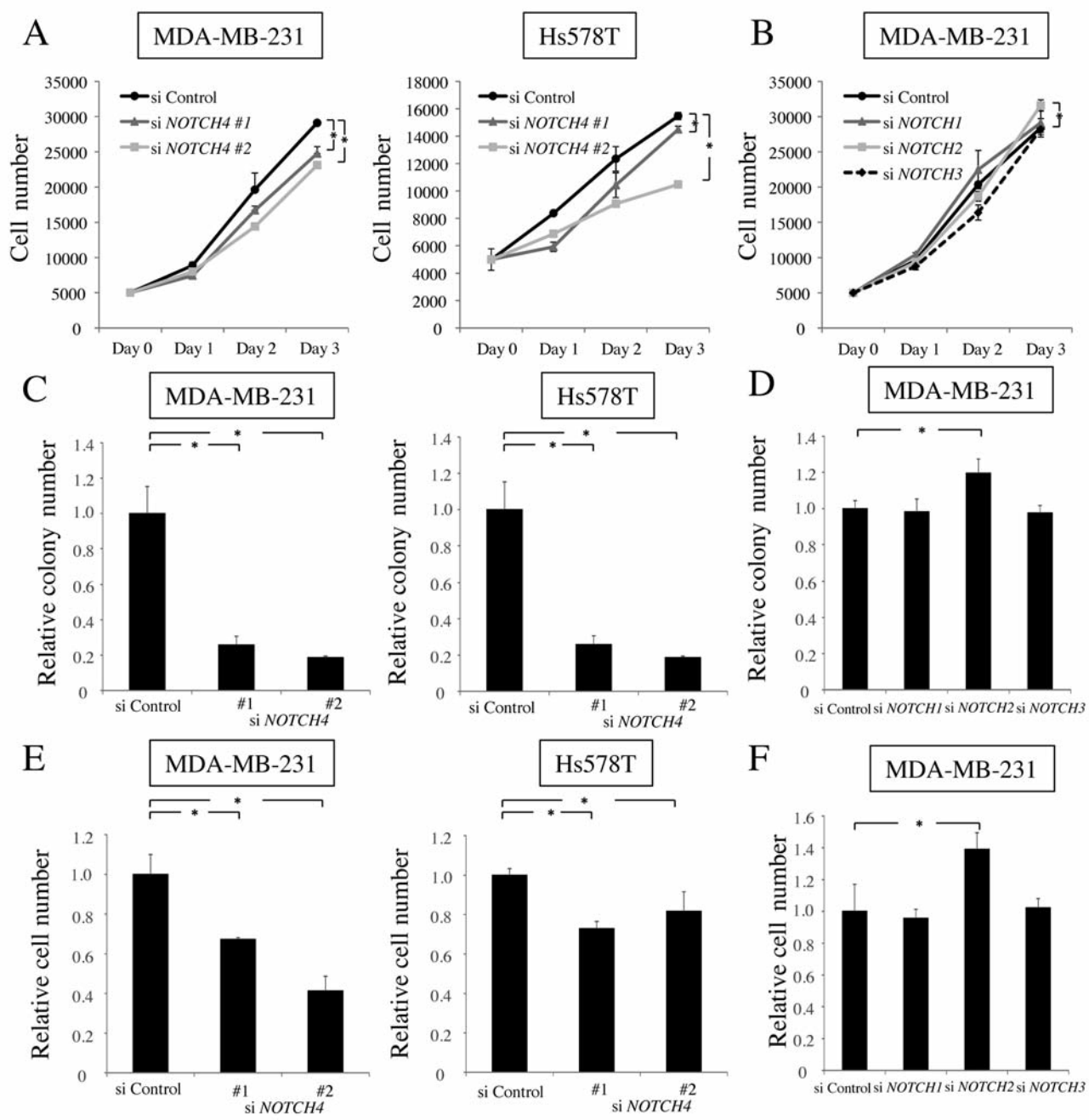


Figure 3. *Inhibition of NOTCH4 reduces proliferation, colony formation and invasiveness in triple-negative breast cancer (TNBC) cells. A: The proliferation of NOTCH4 siRNA-transfected TNBC cells was analyzed using Cell Count Reagent SF. B: The proliferation of NOTCH1, -2 and -3 siRNA-transfected MDA-MB-231 cells was estimated using Cell Count Reagent SF. C, D: TNBC cells were re-suspended in a 0.3% agar solution and overlaid on a 0.3% agar layer without cells. After incubation for 21 days, the colonies were stained with crystal violet and counted under a bright microscope. E: Invasiveness of NOTCH4 siRNA-transfected TNBC cells was investigated using Matrigel invasion assays. F: Invasiveness of NOTCH1, -2 and -3 siRNA-transfected MDA-MB-231 cells was estimated. The graphs show the mean values± SD; *p<0.05.*

4A). The increase in NOTCH4 protein expression in *NOTCH4*-plasmid-transfected TNBC cells was also confirmed by western blotting (Figure 4B). Analysis of anchorage-dependent and -independent proliferation, cell number and colony number in *NOTCH4*-plasmid-transfected TNBC cells indicated that these activities were significantly higher than in control cells (Figure 4C and D). Invasiveness of *NOTCH4*-plasmid-transfected TNBC cells was

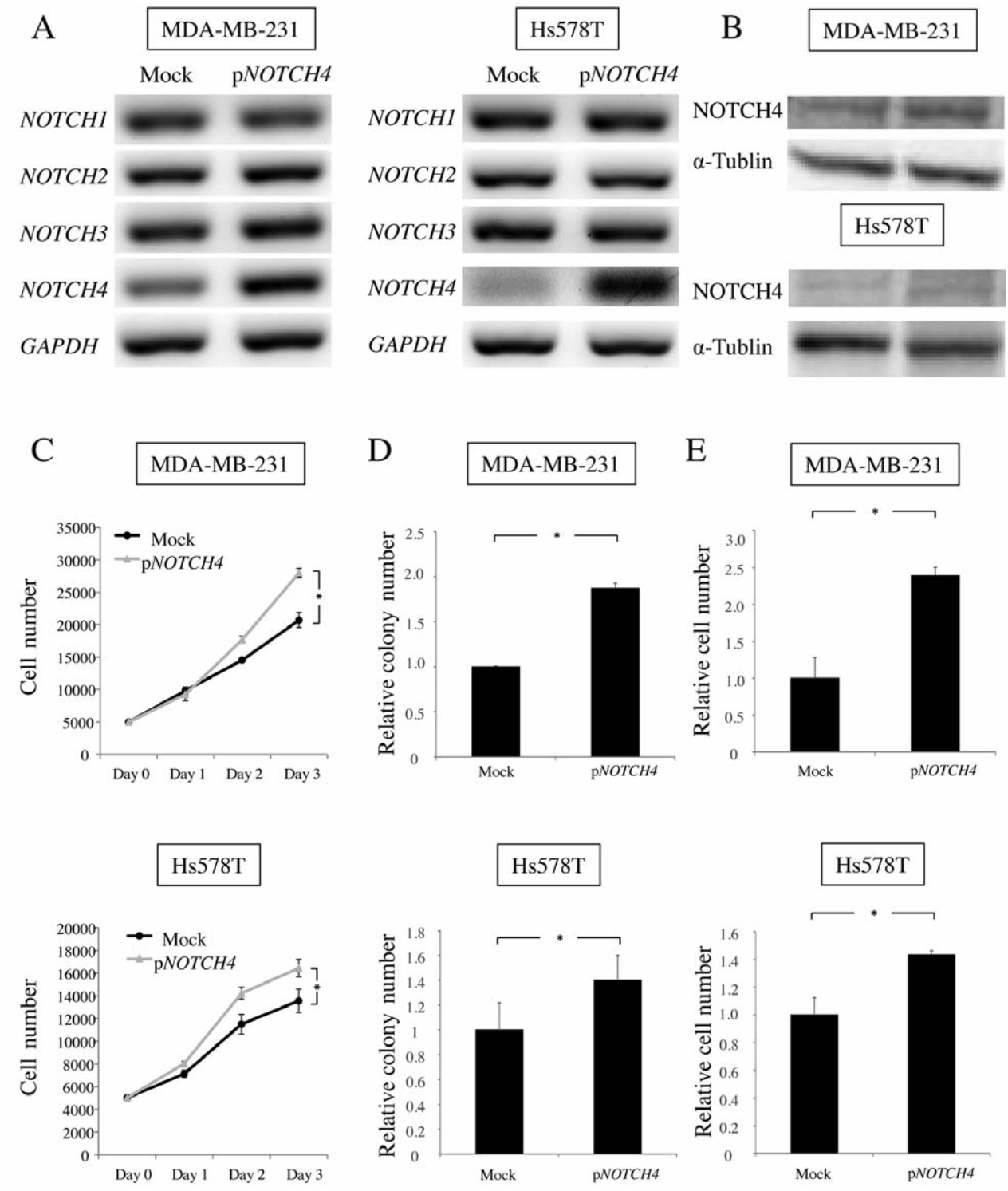


Figure 4. *Overexpression of NOTCH4 increases proliferation, colony formation and invasiveness in triple-negative breast cancer (TNBC) cells. A: Expression of NOTCH1, -2, -3 and -4 mRNAs in NOTCH4 plasmid-transfected TNBC cells was estimated by conventional reverse transcription polymerase chain reaction (RT-PCR). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the loading control. B: Expression of NOTCH4 protein was investigated by western blot analysis. α-Tubulin was used as the loading control. C: Proliferation in NOTCH4 plasmid-transfected TNBC cells was analyzed using Cell Count Reagent SF. D: NOTCH4 plasmid-transfected TNBC cells were re-suspended in a 0.3% agar solution and overlaid on a 0.3% agar layer without cells. After incubation for 21 days, the colonies were stained with crystal violet and counted under a bright microscope. E: Invasiveness in NOTCH4 plasmid-transfected TNBC cells was investigated by Matrigel invasion assays. The graphs show the mean values±SD; * p<0.05.*

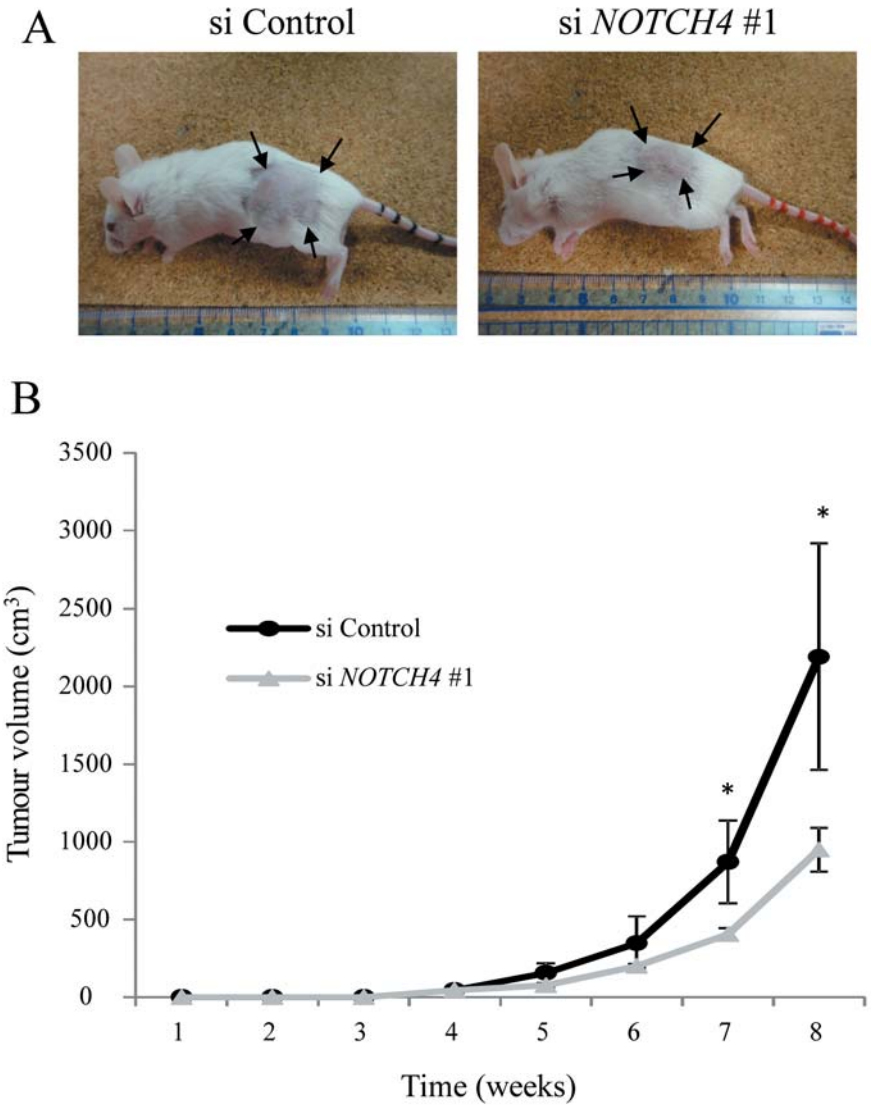


Figure 5. *Inhibition of NOTCH4 suppresses tumorigenicity and tumor volume in a xenograft mouse model. NOTCH4 siRNA- or control siRNA-transfected MDA-MB-231 cells were subcutaneously-implanted into the flank regions of SCID mice at two different doses: 5×10^5 or 1×10^6 cells in PBS per mouse. The tumour size was evaluated once a week. Representative photos are shown. Arrows indicate tumours. The graph shows the mean values±SD; *p<0.05.*

significantly higher than that of control cells (Figure 4E). These results suggest that *NOTCH4* overexpression results in increased proliferation, colony formation and invasiveness of TNBC cells.

Inhibition of NOTCH4 suppresses tumourigenicity and tumour volume in a mouse xenograft model. To determine whether the observed anchorage-dependent and -independent growth in vitro was reflected *in vivo*, MDA-MB-231 cells with or without *NOTCH4* silencing were subcutaneously implanted into the flank regions of SCID mice at two different doses: 1×10^6 cells and 5×10^5 cells. For mice injected with 1×10^6 cells, tumors were detected in all mice in both groups (Table II). In contrast, for those given a dose of 5×10^5 cells, a tumor was detected in one out of eight mice injected with *NOTCH4*-silenced MDA-MB-231 cells, while tumors were confirmed in six out of eight control mice at eight weeks after injection (Table II). Moreover, the tumor

Table II. *Tumourigenesis in a mouse xenograft model.*

Cell number	si Control	si *NOTCH4 #1*	*p*-Value
5×10^5	6/8	1/8	0.012*
1×10^6	6/6	6/6	

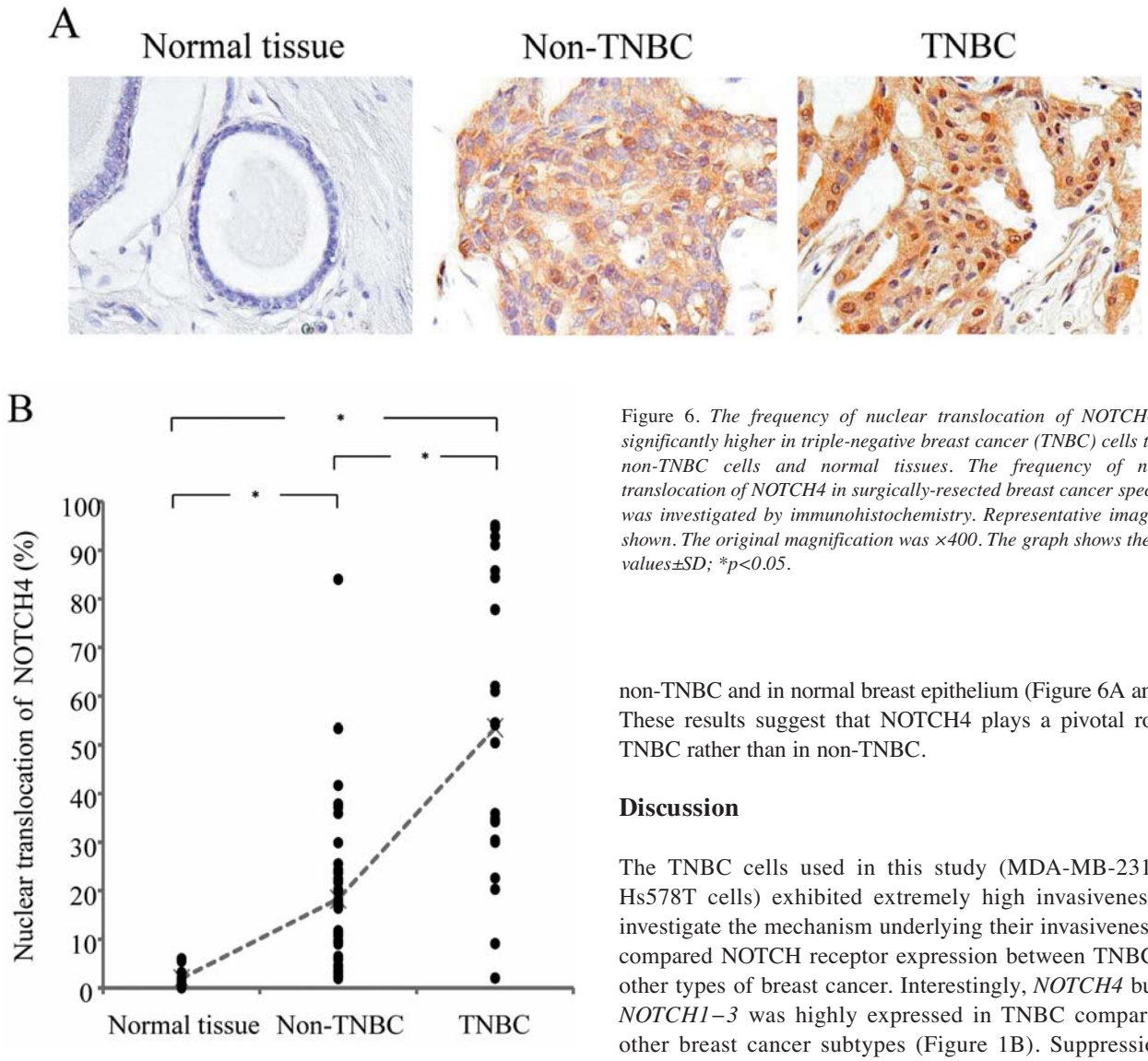


Figure 6. *The frequency of nuclear translocation of NOTCH4 was significantly higher in triple-negative breast cancer (TNBC) cells than in non-TNBC cells and normal tissues. The frequency of nuclear translocation of NOTCH4 in surgically-resected breast cancer specimens was investigated by immunohistochemistry. Representative images are shown. The original magnification was ×400. The graph shows the mean values±SD; *p<0.05.*

volume in mice injected with *NOTCH4*-silenced MDA-MB-231 cells was significantly lower than that in control mice (Figure 5A and B).

The frequency of NOTCH4-intracellular domain (ICD) nuclear translocation was significantly higher in TNBC cells than in non-TNBC and non-cancer cells. To confirm the activation of NOTCH signaling by NOTCH4 receptor in human breast cancer tissue, the frequency of NOTCH4-ICD nuclear translocation in surgically-resected breast cancer specimens was investigated by immunohistochemistry. The clinico-pathological profile is shown in Table III. There were no significant differences in conventional clinicopathological factors between TNBC and non-TNBC cases (Table III). Nuclear translocation of NOTCH4-ICD in TNBC was significantly higher than in non-TNBC and in normal breast epithelium (Figure 6A and B). These results suggest that NOTCH4 plays a pivotal role in TNBC rather than in non-TNBC.

Discussion

The TNBC cells used in this study (MDA-MB-231 and Hs578T cells) exhibited extremely high invasiveness. To investigate the mechanism underlying their invasiveness, we compared NOTCH receptor expression between TNBC and other types of breast cancer. Interestingly, *NOTCH4* but not *NOTCH1–3* was highly expressed in TNBC compared to other breast cancer subtypes (Figure 1B). Suppression of *NOTCH4* dramatically inhibited proliferation, colony formation, invasiveness, and tumourigenesis in TNBC, while inhibition of *NOTCH1–3* had no effect, suggesting that NOTCH4 but not NOTCH1–3 plays a pivotal role in inducing malignant potential in refractory TNBC for which effective therapeutic strategies are limited.

NOTCH signaling was originally associated with cell fate determination, including proliferation and differentiation (23). We therefore investigated the activation of NOTCH signaling by the NOTCH4 receptor in TNBC. Importantly, we observed the nuclear translocation of the NOTCH4-ICD as a marker of activation of NOTCH signaling. NOTCH ICD, which translocates into the nucleus, is released by the proteolytic cleavage of the NOTCH receptors following the activation of NOTCH signaling by the interaction of the receptors and their ligands. Although it was previously reported that the expression of NOTCH receptors is higher

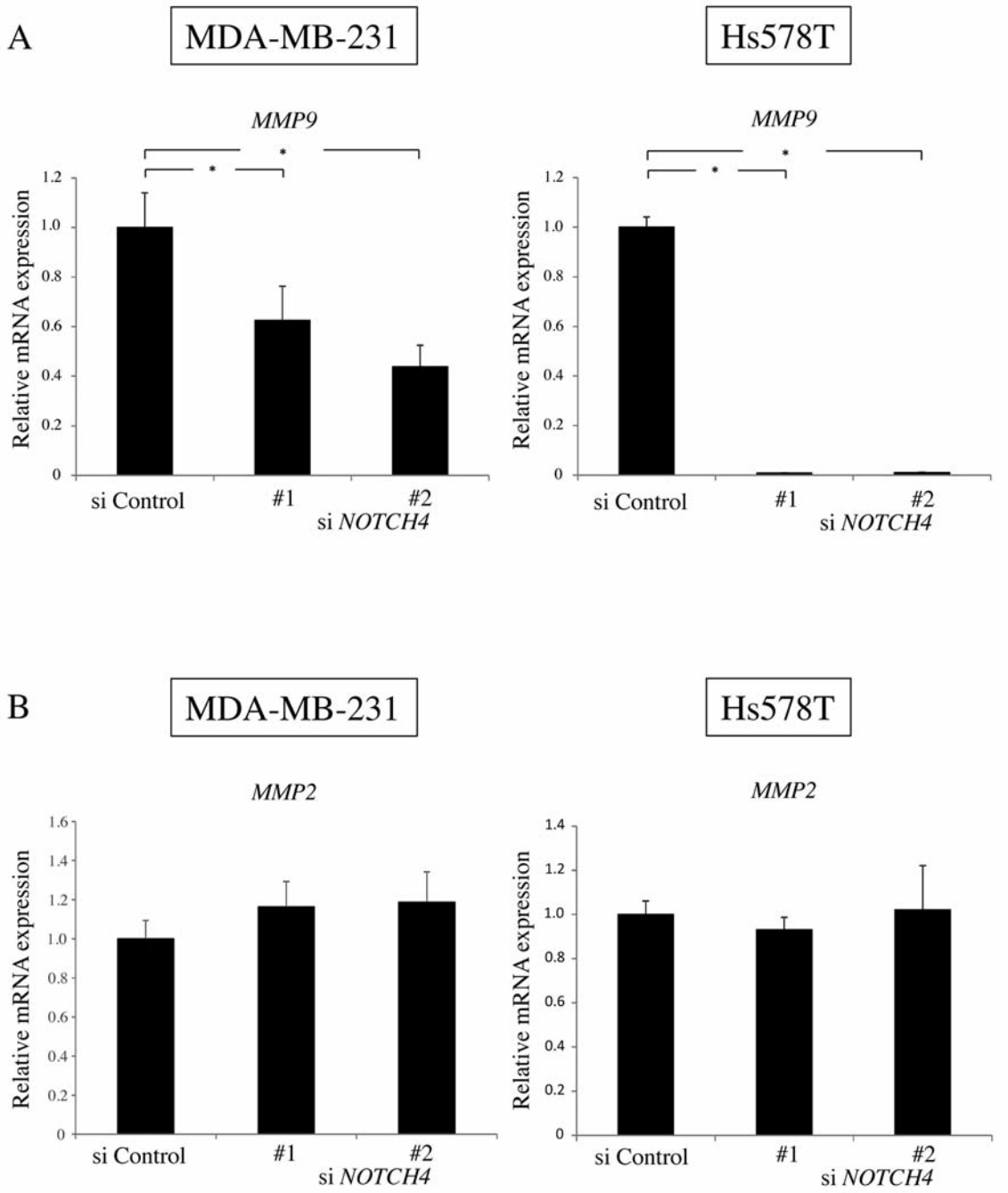


Figure 7. *Inhibition of NOTCH4 reduces matrix metalloproteinase (MMP)-9 mRNA expression in triple-negative breast cancer (TNBC) cells, but does not affect MMP2 mRNA expression in TNBC cells, as estimated by real-time reverse transcription polymerase chain reaction (RT-PCR). A: Expression of MMP9 in NOTCH4 siRNA-transfected TNBC cells was analyzed by real-time RT-PCR. B: Expression of MMP2 mRNA in NOTCH4 siRNA-transfected TNBC cells was analyzed by real-time RT-PCR. The graphs show the mean values±SD; *p<0.05.*

in breast cancer tissue than in normal breast epithelium (24-27), our finding is novel because nuclear translocation of NOTCH4-ICD was observed to be weak in normal breast epithelial cells and strong in cancer tissue (Figure 6A and B). The fact that nuclear translocation of the NOTCH4-ICD was observed in normal breast epithelium suggests that NOTCH4 may have certain biological significance. The high rate of nuclear translocation of the NOTCH4-ICD in cancer tissue suggests that NOTCH4 may also play a pivotal role in the progression and initiation of breast cancer. In addition,

Table III. *Clinicopathological features of 67 invasive breast carcinoma specimens.*

Clinicopathological parameters	Number of specimens		*p*-Value
	Non-TNBC	TNBC	
Age, years			
<50	16	5	0.37
50≥	30	16	
T Classification			
pTl	26	9	0-35
pT2	16	11	
pT3	1	1	
pT4	3	0	
N Classification			
pN0	28	16	0.42
pNl	13	3	
pN2	5	2	
pTNM stage			
I	18	9	0.55
IIA	16	6	
IIB	4	4	
IIIA	5	2	
IIIB	3	0	
ly			
ly0	33	14	0.67
ly1	10	6	
ly2	1	1	
Jy3	2	0	
v			
v0	43	21	0.23
vl	3	0	
Histological type			
Papillotubular carcinoma	18	8	0.2
Solid-tubular carcinoma	4	5	
Scirrhous carcinoma	22	6	
Mucinous carcinoma	2	1	
Apocrine carcinoma	0	1	
Total number of specimens	46	21	

†According to the TNM classification system of the International Union against Cancer. TNM, Tumour node metastasis; TNBC, triple-negative breast cancer.

the fact that the frequency of nuclear translocation of NOTCH4 was higher in TNBC than in other subtypes may support the idea that TNBC may exhibit a more aggressive phenotype than other invasive ductal carcinomas.

In the present study, inhibition of *NOTCH2* induced increased anchorage-dependent and -independent proliferation, and invasiveness (Figure 3B, D, and F). These results are consistent with a report by O'Neill *et al*. that NOTCH2 signaling induced apoptosis and inhibited growth in the MDA-MB-231 xenograft model (28), suggesting NOTCH2 is a potent inhibitory signaling receptor. We will investigate the role of NOTCH2 in future studies.

Recently, Harrison *et al*. demonstrated that the NOTCH4 receptor plays a pivotal role in the control of breast cancer stem cell activity (22). In breast cancer, $CD24^{-/low}CD44^{+}$ is a marker profile for cancer stem cells (29). However, it remains unclear whether the percentage of $CD24^{-/low}CD44^{+}$ cells correlates with cell aggressiveness in breast cancer. The MDA-MB-231 and Hs578T cell lines we used here contain a population of $CD^{24-/low}CD44^{+}$ cells. In our preliminary study, inhibition of *NOTCH4* did not affect the expression of CD24 and CD44 (data not shown). Therefore, we have not yet investigated the relationship between NOTCH4 and breast cancer stem cells. However, this point is important and in our future studies we will address how NOTCH4 may regulate cancer stem cells and cancer progression in TNBC.

To investigate the mechanism underlying decreased invasiveness, the expression of MMP2 and MMP9, type IV collagenases, was investigated. *MMP9* mRNA expression in *NOTCH4* siRNA-transfected TNBC cells was significantly lower than in control cells (Figure 7A). But inhibition of *NOTCH4* did not affect expression of *MMP2* mRNA (Figure 7B). We therefore hypothesize that MMP9 is an important factor for NOTCH4-related invasiveness. The epithelial-to-mesenchymal transition (EMT) is a process that defines how cells lose their polarized epithelial phenotype and acquire a migratory mesenchymal phenotype. EMT is also an important mechanism for invasiveness. Recently, the relationship between NOTCH signalling in breast cancer and EMT has been shown (30). We, therefore, investigated the correlation between NOTCH4-related invasiveness and EMT. However, our preliminary results showed that inhibition of *NOTCH4* did not affect EMT (data not shown). Therefore, it is unlikely that NOTCH4-related invasiveness is mediated through EMT in TNBC.

In conclusion, we showed that NOTCH4, but not NOTCH 1-3, contributes to the induction of malignant potential, including proliferation, tumorigenesis, and invasiveness in TNBC, and that NOTCH4 is a potential therapeutic target for TNBC. Our results may enable new and effective strategies against refractory TNBC to be developed.

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