

Dll4-Fc, an Inhibitor of Dll4-Notch Signaling, Suppresses Liver Metastasis of Small Cell Lung Cancer Cells through the Downregulation of the NF- κ B Activity

Takuya Kuramoto¹, Hisatsugu Goto¹, Atsushi Mitsuhashi¹, Sho Tabata¹, Hirohisa Ogawa², Hisanori Uehara², Atsuro Saijo¹, Soji Kakiuchi¹, Yoichi Maekawa³, Koji Yasutomo³, Masaki Hanibuchi¹, Shin-ichi Akiyama¹, Saburo Sone¹, and Yasuhiko Nishioka¹

Abstract

Notch signaling regulates cell-fate decisions during development and postnatal life. Little is known, however, about the role of Delta-like-4 (Dll4)-Notch signaling between cancer cells, or how this signaling affects cancer metastasis. We, therefore, assessed the role of Dll4-Notch signaling in cancer metastasis. We generated a soluble Dll4 fused to the IgG1 constant region (Dll4-Fc) that acts as a blocker of Dll4-Notch signaling and introduced it into human small cell lung cancer (SCLC) cell lines expressing either high levels (SBC-3 and H1048) or low levels (SBC-5) of Dll4. The effects of Dll4-Fc on metastasis of SCLC were evaluated using a mouse model. Although Dll4-Fc had no effect on the liver metastasis of SBC-5, the number of liver metastasis inoculated with SBC-3 and H1048 cells expressing Dll4-Fc was significantly lower than that injected with control cells. To study the molecular mechanisms of the effects of Dll4-Fc on liver metastasis, a PCR array analysis was conducted. Because the expression of NF- κ B target genes was affected by Dll4-Fc, we conducted an electrophoretic mobility shift assay and observed that NF- κ B activities, both with and without stimulation by TNF- α , were downregulated in Dll4-Fc-overexpressing SBC-3 and H1048 cells compared with control cells. Moreover, Dll4-Fc attenuates, at least in part, the classical and alternative NF- κ B activation pathway by reducing Notch1 signaling. These results suggest that Dll4-Notch signaling in cancer cells plays a critical role in liver metastasis of SCLC by regulating NF- κ B signaling. *Mol Cancer Ther*; 11(12); 2578–87. ©2012 AACR.

Introduction

Small cell lung cancer (SCLC) accounts for 15% to 20% of lung cancer cases and presents with aggressive clinical behavior characterized by rapid growth and metastasis to distant organs (1). The production of metastases in multiple organs such as the liver, bone, and brain during early stages frequently makes the prognosis of patients with these diseases poor. Therefore, novel effective therapies to control cancer metastases are necessary to improve the prognoses of patients with SCLC.

Notch signaling is composed of a family of 4 Notch receptors and 5 ligands. Notch receptors are proteolytically cleaved by γ -secretase upon ligand binding. The

cytoplasmic portions of the receptors then directly transduce signals from the cell surface to the nucleus, thereby controlling the expression of target genes (2). Aberrations of Notch signaling are associated with lung cancer progression as well as T-cell acute lymphoblastic leukemia/lymphoma (T-ALL) and breast cancer progression (3–5). Current evidence provides that VEGF induces Delta-like-4 (Dll4)-regulated differentiation of tip and stalk cells in endothelial cells (6–9). Repression of Dll4-Notch signaling in tumor endothelial cells results in nonproductive angiogenesis and consequent suppression of tumor growth. On the basis of these reports, several Dll4-neutralizing antibodies have been developed that show anticancer effects in preclinical models (10–12). However, the effects of Dll4 expressed by cancer on tumor progression remain to be fully elucidated.

In this study, we investigated the role of Dll4 in metastasis using Dll4-Fc, an inhibitor of Dll4-Notch signaling, and cancer cell lines expressing either high or low levels of Dll4.

Materials and Methods

Cell lines

Human SCLC cell lines, SBC-3 and SBC-5, were kindly provided by Drs. M. Tanimoto and K. Kiura (Okayama University, Okayama, Japan). H1048 cells were purchased

Authors' Affiliations: Departments of ¹Respiratory Medicine & Rheumatology, ²Molecular and Environmental Pathology, and ³Immunology & Parasitology, Institute of Health Biosciences, The University of Tokushima Graduate School, Tokushima, Japan

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Corresponding Author: Yasuhiko Nishioka, Department of Respiratory Medicine and Rheumatology, Institute of Health Biosciences, The University of Tokushima Graduate School, 3-18-15 Kuramoto-cho, Tokushima 770-8503, Japan. Phone: 81-88-633-7127; Fax: 81-88-633-2134; E-mail: yasuhiko@clin.med.tokushima-u.ac.jp

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from the American Type Culture Collection. SCLC cell lines were authenticated by a Multiplex STR assay (BEX). Human umbilical vascular endothelial cells (HUVEC) were purchased from Lonza. The Platinum-E (PLAT-E) retroviral packaging cells were kindly provided by Dr. T. Kitamura (Tokyo University, Tokyo, Japan; ref. 13).

Reagents

Antimouse IL-2 receptor β -chain monoclonal antibody, TM- β 1, was supplied by Drs. M. Miyasaka and T. Tanaka (Osaka University, Osaka, Japan). Antibodies against Notch1, p50, and RelB were purchased from Cell Signaling. Antibodies against p65 and β -actin were purchased from Santa Cruz Biotechnology. Antibody against CD-31 was purchased from BD Biosciences. Antibody against mouse Dll4 was purchased from R&D Systems.

Retroviral transfection

The murine Dll4 extracellular domain-Fc (Dll4-Fc; ref. 6) and its control vector was infected into SBC-3, H1048, and SBC-5 as previously described (14, 15), and infected cell populations were fluorescence-activated cell sorted (FACS) on a JSAN cell sorter (Bay bioscience). The proportion of GFP-positive cells was more than 85% of the cell population.

Animals

Male SCID mice, 5 to 6 weeks of age, were obtained from CLEA Japan and maintained under specific pathogen-free conditions throughout the study. All experiments were conducted in accordance with the guidelines established by the Tokushima University Committee on Animal Care and Use.

In vivo metastasis models

To facilitate metastasis formation, SCID mice were pretreated with TM β -1 to deplete NK cells. Two days later, the mice were inoculated with SBC-3, H1048, or SBC-5 cells into the tail vein (16–18). The mice were sacrificed humanely under anesthesia, and the major organs were then removed and weighed and the number of metastatic nodules on the surface of the organs was counted.

Analysis of micrometastasis

To detect micrometastasis in the liver by reverse transcription (RT)-PCR, whole livers were removed 7, 14, and 21 days after injection of the cells, and RNA was extracted using the RNeasy Mini Kit (Qiagen). Reverse transcription reaction was conducted as follows. RT-PCR was conducted using TaqMan Gene expression assays (Supplementary Table S1). The expression levels of human- β 2-microglobulin (*hB2M*) in the murine livers were used as molecular markers for micrometastasis.

To evaluate the longest diameter or microvessel density in the liver micrometastases, whole livers were removed 21 days after inoculation of SBC-3-Dll4-Fc or control cells. Frozen tissue sections (8- μ m thick) were fixed with 4% paraformaldehyde (PFA) and used to

identify endothelial cells using rat antimouse CD31 monoclonal antibody (1:30 dilution; BD Biosciences). The highest numbers of staining within a section were selected for histologic quantification.

RT-PCR and TaqMan gene signature arrays

Total cellular RNAs were isolated using the RNeasy Mini Kit (Qiagen), and reversely transcribed using a TaqMan RNA-to-CT 2-Step Kit (Applied Biosystems). The primers for *Dll4-Fc* and *β -actin* were as follows: Dll4-Fc: 5'-ACAGGCACCCACTGTGAACT-3' and 5'-CTGGGATGAAGCCTTTGAC-3'; β -actin: 5'-AAGAGAGGCATCCTCACCCCT-3' and 5'-TACATGGCTGGGGTGTGAA-3'. RT-PCR was conducted using Ampli Taq Gold (Applied Biosystems).

A quantitative PCR analysis of metastasis-associated genes was carried out using the TaqMan Array Gene Signature (Applied Biosystems).

Western blot analysis

Cells were cultured for 48 hours, and were lysed in M-PER reagent (Pierce) containing phosphatase and protease inhibitor cocktails (Roche). To detect protein in the conditioned medium, cultured medium was collected after incubation for 48 hours. The metastatic liver lesions were homogenized in T-PER (Pierce). The concentrations of protein were determined using a Bio-Rad Protein Assay Kit (Bio-Rad). Aliquots of 500 μ g of total proteins were immunoprecipitated with the antibody against Notch1 (Cell Signaling Technology). Then, immunoblotting was conducted as previously described (19).

Luciferase assay

The NF- κ B activities in SBC-3, H1048, and SBC-5 cells were measured as previously described (20). SCLC cells were cultured for 48 hours after transfection. Then, the luciferase activities in the cell extracts were measured.

Electrophoretic mobility shift assay

Cells were cultured for 4 hours with the presence or absence of TNF- α (100 ng/mL; R&D Systems). Then, nuclear extractions were prepared using the NE-PER Kit (Pierce). Electrophoretic mobility shift assay was conducted using the Lightshift EMSA Kit (Pierce) according to the manufacturer's protocols. A supershift assay was also conducted to confirm the DNA-NF- κ B bound complexes.

Migration and invasion assay

Cell migration was determined as previously described (21). Invasion assay was conducted using Matrigel-coated transwell chambers (BD Biosciences) according to the manufacturer's protocols. In each analysis, the 4 areas containing the highest number of the cells within a chamber were counted under light microscopy at \times 100 magnification.

Data analysis

The data are expressed as the mean \pm SD. Welch *t* test and the Mann-Whitney *U* test were used for the statistical

analyses. Differences were considered to be significant at $P < 0.05$.

Results

Retroviral transduction of Dll4-Fc in cancer cells suppresses Dll4-Notch signaling

On the basis of previous studies that used extracellular domain of Dll4 to inhibit Dll4-Notch signaling (6), we generated a retroviral vector encoding the extracellular domain of murine Dll4 fused to the human IgG1 Fc constant region (Dll4-Fc) with GFP. This construct was infected into human SCLC cell lines expressing either high levels of Dll4 (SBC-3 and H1048) or low levels of Dll4 (SBC-5; Supplementary Fig. S1A). The Dll4-Fc-transduced cancer cells were sorted with flow cytometry (FACS). *Dll4-Fc* mRNA was detected in the Dll4-Fc-transduced SBC-3 cells (SBC-3-Dll4-Fc) but not in the vector-transduced control cancer cells (Fig. 1A). Dll4-Fc proteins were detected in the cell lysate and conditioned medium of the SBC-3-Dll4-Fc cells but not in those of the control cells (Fig. 1B). Similar results were obtained in the H1048 and SBC5 cells (Supplemental Fig. S1B, S1C, S1E, and S1F).

To examine whether Dll4-Fc affected the behavior of cells, the growth rates of cells were examined using an MTT assay. The growth rates of the SBC-3-Dll4-Fc, H1048-Dll4-Fc, and SBC5-Dll4-Fc cells were similar to those of the counterpart of control cells (Fig. 1C and Supplementary Fig. S1D and S1G). We then examined whether transduced Dll4-Fc inhibits Dll4-Notch signaling. Confluent HUVECs were treated with the conditioned medium of the SBC-3-Dll4-Fc cells and the *Hes1* expression was measured as a surrogate marker of Notch activity. The conditioned medium of the SBC-3-Dll4-Fc cells suppressed the *Hes1* expression (Fig. 1D). This result indicates that the Dll4-Fc secreted from SBC-3-Dll4-Fc cells is functional.

Dll4-Fc suppresses liver metastasis of SCLC cells expressing high levels of Dll4

We examined whether Dll4 plays an important role in cancer metastasis using NK-cell-depleted SCID mice as a multiple-organ metastasis model. In this model, SBC-3 and H1048 cells expressing high levels of Dll4 metastasized to the liver, kidney, bone, and lymph node (17, 18). We observed that SBC-3-Dll4-Fc and H1048-Dll4-Fc cells produced significantly fewer numbers of metastatic nodules in the liver compared with the control cells (Table 1). The numbers of metastatic nodules in other organs, such as the kidney, bone, and lymph node were not affected by Dll4-Fc. The mice intravenously inoculated with SBC-5 cells expressing low levels of Dll4 had liver, lung, and bone metastases (16). The numbers of metastatic nodules produced by SBC-5-Dll4-Fc cells in those organs were comparable with those produced by control SBC-5 cells (Table 2). These results suggest that Dll4 reveals prometastatic function in the liver in proportion to its expression.

Dll4-Fc suppresses liver micrometastasis of SCLC cells

The number of macroscopic metastatic nodules produced by the SBC-3-Dll4-Fc cells in the liver was less than that produced by the control cells; however, the size of the nodules produced by the SBC-3-Dll4-Fc cells was comparable with that of the nodules produced by the control cells (Fig. 2A). Furthermore, the proportion of metastatic nodules larger than 2 mm produced by the SBC-3-Dll4-Fc cells in the liver was comparable with that produced by the control cells (Fig. 2B). These results suggest that Dll4-Fc affects the early steps of metastasis such as survival in circulation, attachment to endothelial cells, migration, invasion, and extravasation, but not growth or neovascularization. To confirm this hypothesis, we evaluated

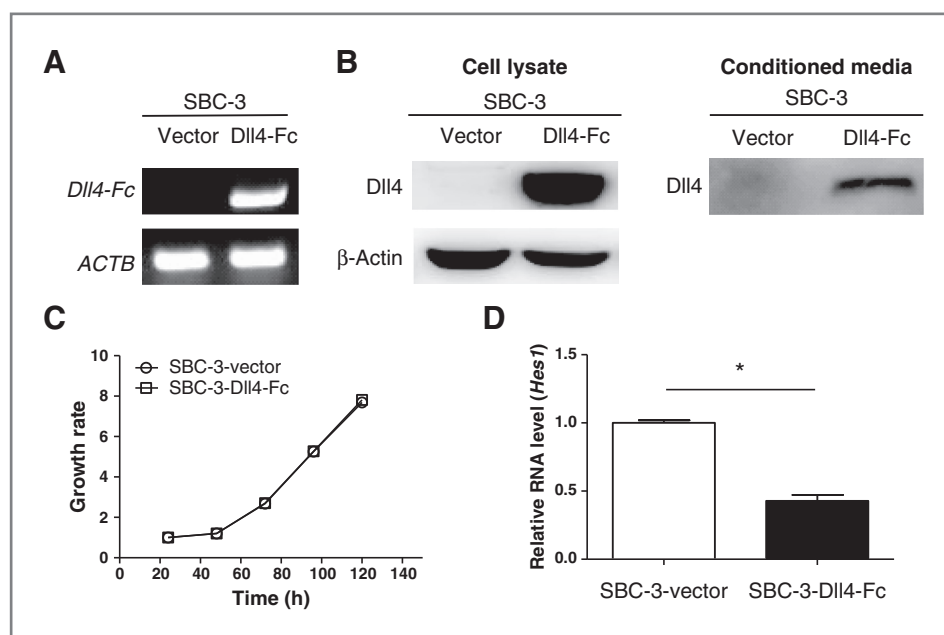


Figure 1. Extracellular domain of murine Dll4 fused to Fc (Dll4-Fc) expressed in cancer cells inhibits Notch signaling. A and B, the expression of *Dll4-Fc* mRNA (A) and Dll4-Fc protein in SBC-3 cells (B, left) and conditioned media (B, right) was determined. C, the effects of Dll4-Fc on the growth rates of the SBC-3 cells were measured using an MTT assay. D, the effects of the conditioned media of the SBC-3-vector and SBC-3-Dll4-Fc on *Hes1* mRNA expression in HUVECs; *, $P < 0.05$ (Welch *t* test).

Table 1. Production of metastasis by SCLC cells expressing high level of DII4 with or without DII4-Fc in NK cell-depleted SCID mice

Cell line	Incidence	Liver weight (g)	No. of metastatic colonies ^a		
			Liver	Kidney	Lymph node
SBC-3-vector	7/7	2.6 (1.6–5.4)	15.6 (2–43)	4.0 (0–11)	2.1 (1–3)
SBC-3-DII4-Fc	9/9	1.3 (1.1–1.5) ^b	4.0 (1–8) ^c	2.8 (0–5)	2.0 (1–3)

Cell line	Incidence	Liver weight (g)	No. of metastatic colonies ^a		
			Liver	Kidney	Bone
H1048-vector	6/6	1.1 (0.8–1.4)	4.8 (2–7)	59.8 (39–77)	9.5 (8–12)
H1048-DII4-Fc	7/7	0.8 (0.6–1.1)	1.6 (1–3) ^d	49.9 (19–84)	7.7 (3–14)

NOTE: The statistical significance of difference was analyzed by one-way ANOVA and *post hoc* pairwise comparisons were done by Newman-Keuls multiple comparison test.

^aValues are the mean (minimum–maximum).

^bStatistically significant difference compared with SBC-3-vector ($P < 0.05$).

^cStatistically significant difference compared with SBC-3-vector ($P < 0.001$).

^dStatistically significant difference compared with H1048-vector ($P < 0.05$).

micrometastasis of SBC-3-DII4-Fc and control cells in the liver to investigate the effects of DII4-Fc on the early steps of SCLC metastasis. To detect micrometastasis, whole livers were removed on days 7, 14, and 21 after injection of the cells and the expression of hB2M as a molecular marker of liver metastasis was detected using RT-PCR. As shown in Fig. 2C, the levels of hB2M mRNA in the livers of mice inoculated with SBC-3-DII4-Fc cells were significantly lower than those in the livers of mice inoculated with control cells, on day 21, suggesting that DII4-Fc plays a role in the early steps of metastasis produced by SCLC cells (Fig. 2C). A histologic analysis was also done on day 21 using metastatic liver tissue after inoculation with either SBC-3-DII4-Fc or control cells. All of the metastatic foci in the liver were smaller than 1.5 mm in diameter (Fig. 2D and E), and the diameters of the metastatic foci produced by the SBC-3-DII4-Fc cells in the liver were comparable with those of the metastatic foci produced by the control cells (Fig. 2E). As a previous report showed that the DII4-Fc suppresses tumor growth by promoting dysregulated angiogenesis (6), we evaluated the effects of DII4-Fc on tumor angiogenesis in metastatic foci of the

liver on day 21. The microvessel density in the micro-metastatic foci produced by the SBC-3-DII4-Fc cells was comparable with that in the micrometastatic foci produced by the control cells (Fig. 2F and G), thus indicating that DII4-Fc does not affect angiogenesis in metastatic foci. Taken together, these *in vivo* results suggest that blockage of DII4-Notch signaling in cancer cells suppresses the early steps of liver metastasis produced by SBC-3 cells.

DII4-Fc reduces migration ability and invasiveness

Cancer metastasis is a multistep process requiring tumor cell migration, intravasation, survival in circulation, extravasation, and colonization to a secondary site. Especially, the activities of migration and invasion play important roles in the early steps of metastasis. To examine the effect of DII4-Fc on the cell migration, migration assay was conducted using SBC-3 and SBC-5 cells expressing DII4-Fc. Although the cell migration of the SBC-5 expressing DII4-Fc was comparable with that of the control cells (data not shown), the migration ability of the SBC-3 cells was suppressed by DII4-Fc (Fig. 3A and B). Moreover, to evaluate the effects of DII4-Fc on the ability

Table 2. Production of metastasis by SCLC cells expressing low level of DII4 with or without DII4-Fc in NK cell-depleted SCID mice

Cell line	Incidence	Liver weight (g)	No. of metastatic colonies ^a		
			Liver	Lung	Bone
SBC-5-vector	8/8	1.4 (0.7–2.0)	43.8 (24–91)	54.3 (8–90)	4.0 (1–5)
SBC-5-DII4-Fc	9/9	1.3 (1.1–1.5)	30.8 (15–68)	69.7 (47–102)	2.8 (1–5)

NOTE: The statistical significance of difference was analyzed by one-way ANOVA and *post hoc* pairwise comparisons were done by Newman-Keuls multiple comparison test.

^aValues are the means (minimum–maximum).

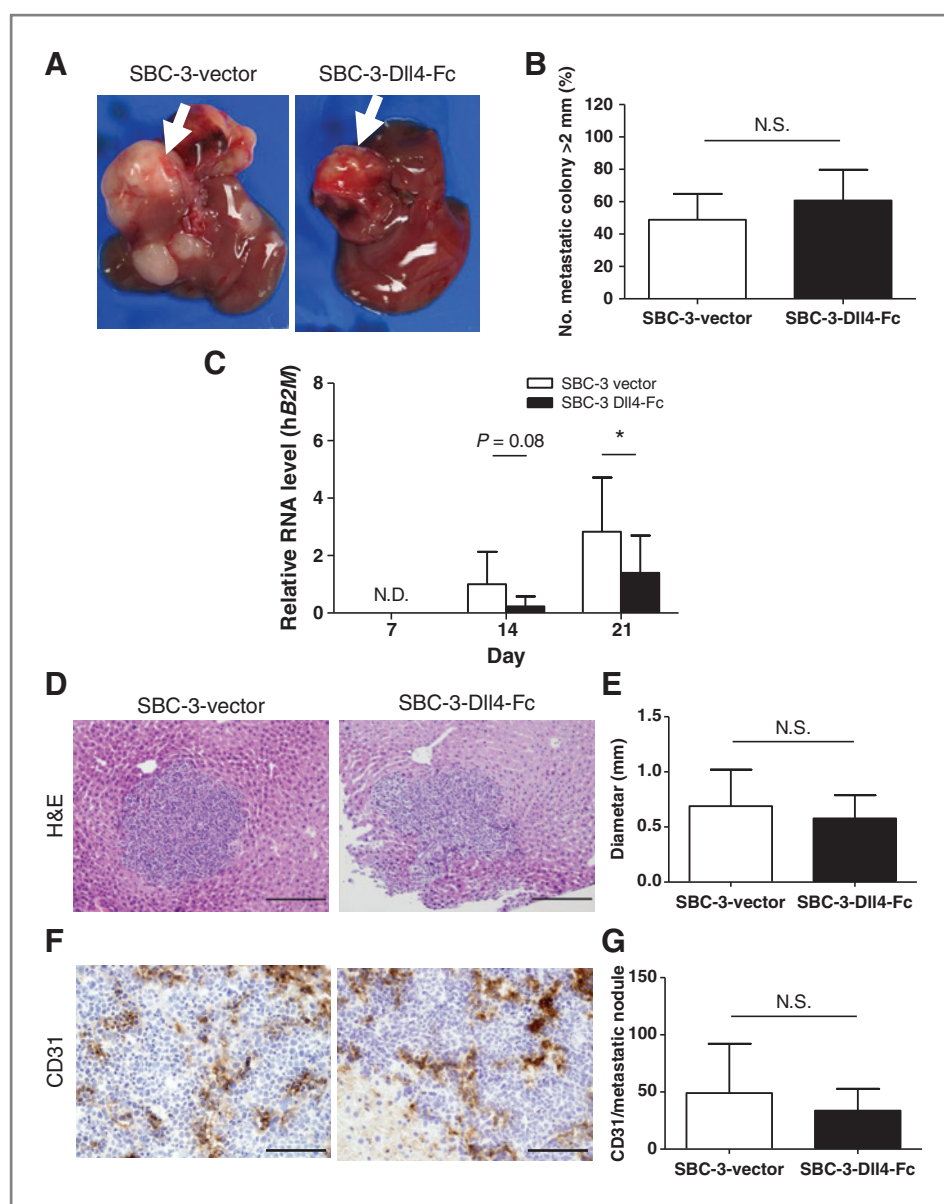


Figure 2. Dll4-Fc suppresses micrometastasis of SBC-3 cells in the liver. **A**, representative macroscopic metastatic nodules on the surface of the livers 42 days after inoculation of control (left) and SBC-3-Dll4-Fc cells (right) in SCID mice are shown. The size of the tumor produced by SBC-3-Dll4-Fc cells was comparable with that of the tumor produced by control cells (arrow). **B**, the proportion of metastatic nodules larger than 2 mm on the surface of the liver was measured 42 days after inoculation of control and Dll4-Fc-expressing SBC-3 cells. **C**, micrometastasis in the liver was assessed by RT-PCR. The relative expression was calculated according to the expression of hB2M 14 days after inoculation of SBC-3-vector as 1.0. **D** and **E**, the longest diameter of each metastatic foci in the liver was measured after hematoxylin and eosin staining. **D**, photographs of the representative metastatic foci of SBC-3-vector and SBC-3-Dll4-Fc cells are shown (H&E; magnification, $\times 40$). **E**, the longest diameter of each metastatic foci was measured and plotted. Bar, 1.0 mm. **F** and **G**, staining of the microvessels in the metastatic foci in the liver with antimouse CD31 monoclonal antibody 21 days after injection of SBC-3-Dll4-Fc and control cells. **F**, representative CD31 staining patterns. **G**, the analysis of the number of microvessels in the metastatic foci. Bar, 200 μm ; *, $P < 0.01$ (Mann-Whitney U test); N.D., not detected; N.S., not significant.

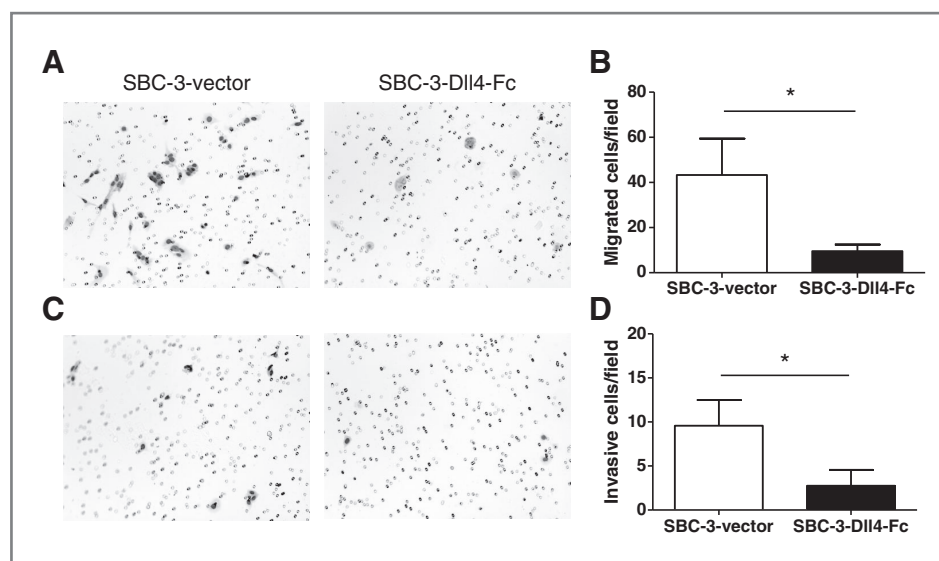
of the invasion, we conducted an invasion assay using Matrigel-coated Transwell chambers (Fig. 3C and D). The invasion activity of the SBC-3 cells was also attenuated by Dll4-Fc. These results suggest that Dll4-Fc inhibits the early steps of liver metastasis through the attenuation of cell migration and invasion.

Dll4-Fc suppresses the expression of metastasis-associated genes through the inhibition of NF- κ B activity

To elucidate the molecular mechanisms underlying the effects of Dll4-Fc on liver metastasis, the expression levels of genes associated with metastasis were determined using a PCR array system. Six genes (*SERPINE1*, *MMP10*, *CXCR4*, *MMP7*, *S100A4*, and *MMP1*) were downregulated in Dll4-Fc-overexpressing SBC-3 and H1048 cells,

but not in SBC-5 cells, compared with control cells (Fig. 4A and Supplementary Table S2). Several signaling pathways were involved in these genes. However, because the most selected genes, *SERPINE1*, *CXCR4*, *S100A4*, and *MMP1*, were regulated by NF- κ B, we explored whether NF- κ B was involved in Dll4-Notch signaling in these cells (22–25). By conducting a luciferase assay using a luciferase reporter plasmid carrying 6 tandem NF- κ B binding sites, we observed that the level of NF- κ B activity was decreased in Dll4-Fc-overexpressing SBC-3 and H1048 cells, but not in SBC-5 cells, compared with that observed in control cells (Fig. 4B). In addition, to confirm the suppression of NF- κ B activity by Dll4-Fc, the effects of recombinant Dll4-Fc on NF- κ B activity of SBC-3-vector cells were measured by luciferase assay (Supplementary Fig. S2). Similar results were observed with SBC-3-Dll4-

Figure 3. DII4-Fc suppresses the migration and invasion abilities in SBC-3 cells. The effects of DII4-Fc on the migration and invasion abilities of SBC-3 cells were measured using a Transwell assay. A and C, representative stainings of migrated (A) and invasive (C) cancer cells are shown. B and D, the cells that migrated (B) and invaded (D) to the lower surface of the insert were counted; *, $P < 0.01$ (Welch t test).



Fc. To explore the mechanisms underlying the attenuation of NF- κ B signaling by DII4-Fc, we conducted an Electrophoretic Mobility Shift Assay (EMSA). Although we could not detect a specific DNA-protein complex in the nuclear extracts of the SBC-5 cells (data not shown), 2 or 3 complexes were detected in the nuclear extracts of the SBC-3 and H1048 cells, which were, at least in part, reduced by DII4-Fc (Supplementary Fig. S3A and S3B). Because the H1048 cells showed higher expression levels of *TNFA* mRNA than the SBC-3 cells (data not shown), we suggested that DII4 affected NF- κ B activity either with or without TNF- α . To confirm this hypothesis, we conducted EMSA with stimulation of TNF- α (Fig. 4C). In accordance with Fig. 4B, we represented that DII4-Fc, at least in part, suppressed basal level of DNA-bound complexes (Fig. 4C and Supplementary Fig. S3C; lane 1, 6, 8, and 13). Moreover, we found that the upper DNA-bound complex of the SBC-3 cells was elevated with TNF- α treatment (Fig. 4C and Supplementary Fig. S3C; lanes 1 and 2) and partially suppressed by DII4-Fc (Fig. 4C and Supplementary Fig. S3C; lanes 2 and 7). Similar results were obtained with stimulation of the H1048 cells by TNF- α (Fig. 4C and Supplementary Fig. S3C; lanes 9 and 14). To reveal the molecular mechanisms underlying suppression of NF- κ B signaling by DII4-Fc with TNF- α treatment, we examined supershifts using antibodies against p65 and Notch1. The upper complexes elevated by TNF- α were attenuated when antibody against p65 was added to the SBC-3 and H1048 cells (Fig. 4C and Supplementary Fig. S3C; lanes 2, 4, 9, and 11). In addition, all complexes were partially suppressed by antibody against Notch1 (Fig. 4C and Supplementary Fig. S3C; lanes 2, 5, 9, and 12). These results imply that DII4-Notch signaling is involved in NF- κ B activity both with and without TNF- α stimulation. Although antibody against Notch1 partly suppressed the upper, middle, and bottom of DNA-bound complexes, antibody against p65 only attenuated upper bound complex with TNF- α stimulation. To determine the lower 2

bound complexes, we conducted supershifts using antibodies against p50 and RelB (Fig. 4D). The upper and bottom DNA bound complexes were reduced by antibody against p50, and middle band was attenuated by antibody against RelB. These results suggested that p65, p50, and RelB were interacted with Notch1. To confirm these results, we examined the interaction of Notch1 with p65, p50, and RelB in 3 nodules of the liver metastasis produced by SBC-3 (Fig. 4E). We found that Notch1 bound to p65, p50, and RelB in metastatic liver lesions. Moreover, DII4-Fc attenuated these interactions. To investigate the effects of DII4-Fc on Notch1 signaling, we measured the cleavage of the Notch1 intracellular domain (N1-ICD) as a marker of Notch1 activity in DII4-Fc expressing SBC-3, H1048, and SBC-5 cells. An immunoblot analysis showed that the levels of N1-ICD was reduced in DII4-Fc-transduced SBC-3 and H1048 cells, but not in DII4-Fc-transduced SBC-5 cells, compared with that observed in control cells (Fig. 4F). These results indicate that a reduction of N1-ICD induced by DII4-Fc attenuates the binding of p65, p50, and RelB to DNA and consequently reduces the level of NF- κ B activity. Moreover, we determined the levels of migration activity of SBC-3 expressing DII4-Fc and control cells stimulated by TNF- α (Fig. 4G). In accordance with Fig. 3B, DII4-Fc suppressed the migration ability of SBC-3 cells. In addition, we found that the migration ability of SBC-3 cells was elevated with TNF- α treatment and significantly reduced by DII4-Fc. These results suggest that downregulation of NF- κ B activity induced by the reduction of N1-ICD is involved in the inhibition of liver metastasis through the repression of cell migration.

Discussion

In this study, we showed that DII4-Fc inhibits the liver metastasis caused by the suppression of early steps of metastasis in SCLC cells expressing high levels of DII4 (Supplementary Fig. S1A, Table 1 and Fig. 2C). Moreover,

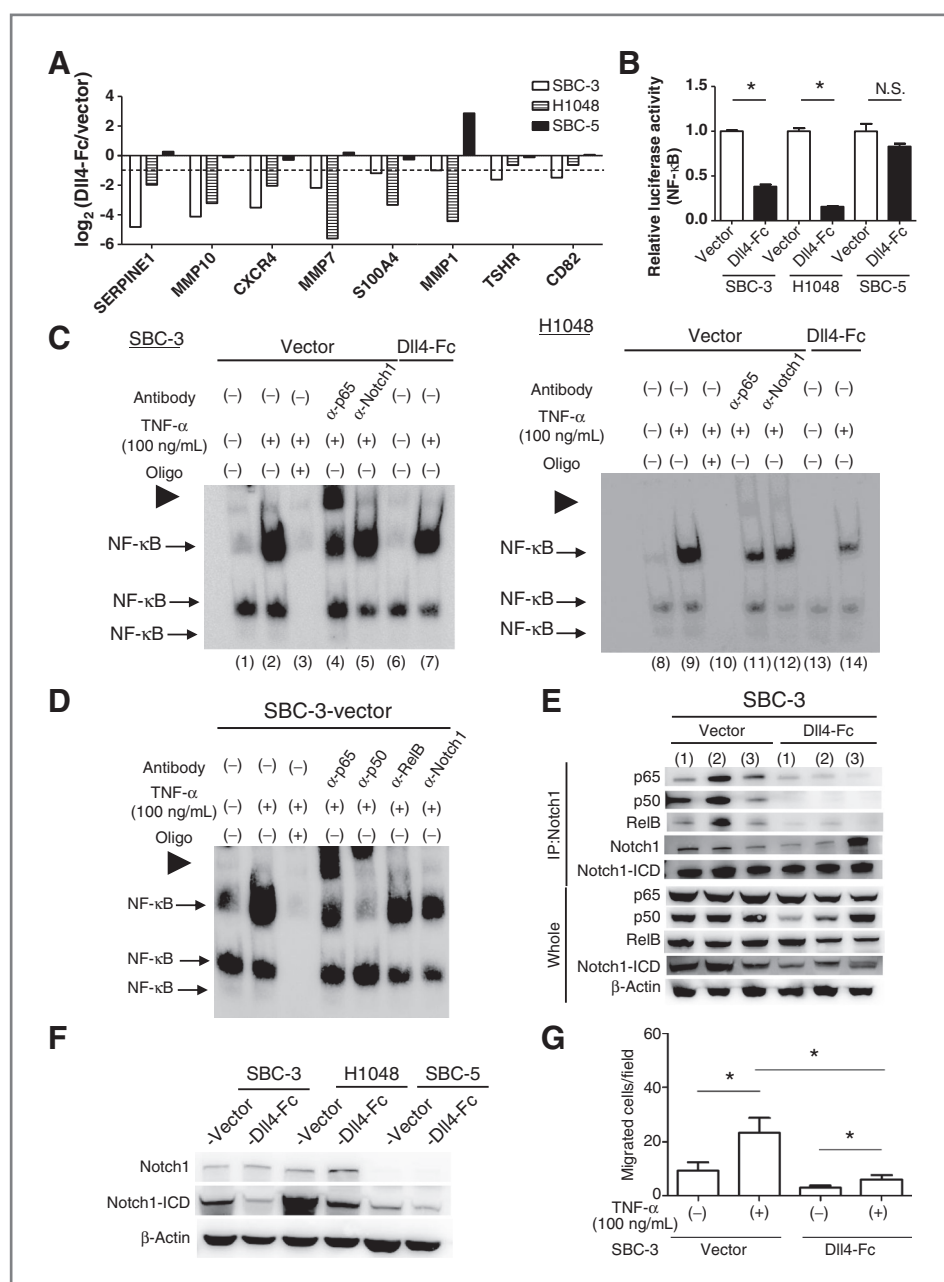


Figure 4. Dll4-Fc suppresses NF- κ B activity via prevention of Notch signaling. **A**, the expression of metastasis-associated genes of SBC-3, H1048, and SBC-5 were measured using RT-PCR array systems. The data represent differences (\log_2 fold changes) in the expression levels of the genes between Dll4-Fc-expressing and control cells. The dashed line indicates \log_2 (fold changes) of -1.0 . **B**, the effects of Dll4-Fc on NF- κ B activity in SBC-3, H1048, and SBC-5 cells were determined using a luciferase assay. **C**, EMSAs were conducted on nuclear extracts of SBC-3 (left) and H1048 (right) cells. Competition assays were done with excesses of unlabeled NF- κ B oligonucleotides (oligo). **D**, a supershift assay using antibodies against p65, p50, RelB, and Notch1 were conducted to confirm the DNA-bound complexes in the SBC-3-vector cells. **C** and **D**, DNA-bound complexes were identified (arrow). The arrowhead indicates the supershift. **E**, the protein extractions of liver metastasis produced by SBC-3 cells were immunoprecipitated (IP) with anti-Notch1 antibody, and immunoprecipitates or total extractions were immunoblotted. **F**, the levels of intracellular domain (ICD) of Notch1 were measured by immunoblotting. **G**, the effects of Dll4-Fc on the migration ability of SBC-3 cells treated with TNF- α (100 ng/mL) were measured using Transwell; *, $P < 0.01$ (Welch t test).

Dll4-Fc attenuated, at least in part, the classical and alternative NF- κ B activation pathway by reducing Notch1 signaling (Fig. 4). These findings highlight the importance of Dll4-Notch1 signaling in liver metastasis from lung cancer.

Several studies showed that VEGF-induced Dll4 in tip cells regulated the formation of stalk cells (6–9). Noguera-Troise and colleagues revealed that blockage of Dll4-Notch signaling by Dll4-Fc in endothelial cells suppressed tumor growth by promoting nonproductive angiogenesis. In accordance with these reports, we showed that suppression of Dll4-Notch signaling by Dll4-Fc in cancer cells resulted in increase of endothelial cell density in liver metastasis produced by SBC-3 cells (Supplementary Fig. S4A and S4B).

However, the number of endothelial cells in liver metastasis produced by H1048-Dll4-Fc cells tended to be higher than that of control cells, whereas the difference was statistically not significant (Supplementary Fig. S4C and S4D). Because the transduction of Dll4-Fc did not affect VEGFA expression and its receptor activity of HUVECs (Supplementary Fig. S4E and S4F), these results suggest that the suppression of Dll4-Notch signaling by Dll4-Fc in cancer cells may have an effect to promote nonproductive angiogenesis, but the effect may vary depending on the tumor microenvironment. However, this study indicates that Dll4-Fc suppresses the early steps of liver metastasis of the SBC-3 cells without affecting angiogenesis, implying that Notch signaling of cancer cells stimulated by adjacent cancer cells

derived from DII4 is implicated in metastasis. A previous report showed that DII4 expressed in tumor cells regulated cancer growth and differentiation (26), and improved tumor vascular function and promoted tumor growth (27, 28). Moreover, Zhang and colleagues showed that DII1-Notch signaling in cancer cells regulates invasion and metastasis *in vitro* and *in vivo* (29). These results suggest that DII4-Notch signaling in cancer cells may be involved in the progression of cancer. Recent evidence showed that Notch signaling between cancer and stromal cells also play an important role in metastasis. Sonoshita and colleagues showed that stromal DII4 and Jagged1 facilitated local tumor invasion and intravasation through the inhibition of amino-terminal enhancer of split (AES) as an endogenous metastasis suppressor (30). Although the current experiments did not reveal the effects of stromal-derived DII4 on metastasis, these findings indicate that the activation of Notch signaling of cancer cells stimulated by adjacent cancer and stromal cells expressing DII4 is implicated in metastasis.

NF- κ B plays an important role in lung cancer progression, including metastasis (31). In this study, we showed that DII4-Notch1 axis regulated NF- κ B signaling irrespective of TNF- α stimulation. Several reports described the interactions between Notch and NF- κ B signaling in normal and cancer cells; however, the underlying molecular mechanisms remain unclear (32). Previous studies showed that downregulation of Notch1 inhibited invasion of pancreatic cancer cells by inactivation of classical NF- κ B (33). These studies showed that knockdown of Notch1 reduced p65/DNA binding activity. In accordance with these results, we showed that DII4-Fc suppressed the NF- κ B activity, and attenuated the migration and invasion abilities (Figs. 3 and 4). Moreover, Shin and colleagues revealed that Notch1 enhanced NF- κ B activity by facilitating nuclear retention (34). They showed that a direct interaction between N1-ICD and NF- κ B subunits can override the cytoplasmic sequestration of NF- κ B by I κ B proteins by promoting nuclear retention of NF- κ B. Although we indicated interaction between N1-ICD and p65, the retention of NF- κ B subunits in the nuclei was not detected irrespective of TNF- α stimulation (Supplementary Fig. S5). These discrepancies might be because of differences in the types of cells and ligands of stimulation. Although further studies are required to clarify the mechanism, these findings indicate that interactions of Notch1 with p65 and p50 with stimulation of TNF- α may play an important role in the formation of metastasis of SCLC. In addition, we found that DII4-Notch1 signaling was involved in the alternative NF- κ B activation pathway through the interaction of RelB. The role of alternative NF- κ B activation pathway in cancer cells remained unclear. Several reports showed that Notch1 regulated the expression of p52 and RelB (35, 36). Because, as far as we know, it is the first report to show the interaction of Notch1 and RelB, these findings may be a novel mechanism for regulation of the alternative NF- κ B activation pathway by Notch1.

In this study, we showed that DII4-Fc suppressed cancer cell migration, but not cell growth or proliferation, through the downregulation of NF- κ B. However, several reports showed that inhibition of NF- κ B reduced tumor cell growth and proliferation (37). These discrepancies might be due to differences in the type of cells. We indicated that SBC-3 and H1048 cells showed weak activation of NF- κ B activity without any stimulation, suggesting that the growth of these cells might not be dependent on the activation of NF- κ B *in vitro* (Fig. 4C and Supplementary Fig. S3C).

DII4-Fc suppressed metastasis in the liver but not in the kidney, lymph node, or bone. It remained unclear why DII4-Fc preferentially suppressed liver metastasis (Table 1). In this study we showed that DII4-Fc suppressed the CXCR4 expression in SBC-3 and H1048 cells (Fig. 4A). CXCR4 is a crucial gene belonging to the chemokine-receptor super family associated with the preference of metastatic sites (38). The CXCL12-CXCR4 axis is involved in regulating the liver, adrenal gland, bone marrow, and brain metastasis of non-small cell lung cancer, and the expression level of CXCL12 in the liver is higher than that in the kidney (39). The attenuation of the expression of CXCR4 by DII4-Fc may cause an obvious suppression of liver metastasis. Further study is needed to elucidate the detailed mechanisms underlying the preferential suppression of liver metastasis by DII4-Fc.

In summary, we have shown that DII4-Fc suppressed liver micrometastasis of cancer cells expressing high levels of DII4. In addition, we found that the microvessel densities of metastatic foci in the liver 21 days after inoculation of SBC-3-DII4-Fc cells were comparable with those of the metastatic foci in control cells. The suppression of liver metastasis by DII4-Fc may be caused by the attenuation of the classical and alternative activation pathway of NF- κ B signaling through the inhibition of the Notch1 activity. Whether other Notch receptors implicate in the metastasis of SCLC, and which NF- κ B pathways regulated by Notch1 are implicated in metastasis of SCLC requires further investigations. However, because several genes expression was specifically regulated in each of metastatic lesion (40), suggesting that the elucidation of mechanisms of each organ metastasis is necessary to develop effective therapies to control cancer metastasis, our findings could be useful for therapy of SCLC patients with multiorgan metastasis. Many Notch inhibitors and antibodies against DII4 have been emerging as novel anticancer drugs. They may therefore be efficacious against liver metastasis in SCLC patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: T. Kuramoto, H. Goto, S. Sone, Y. Nishioka
Development of methodology: T. Kuramoto, H. Goto, A. Mitsuhashi, H. Ogawa, A. Saijo
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T. Kuramoto, H. Goto, S. Tabata, Y. Nishioka

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): T. Kuramoto, H. Goto, S. Tabata
Writing, review, and/or revision of the manuscript: T. Kuramoto, H. Goto, M. Hanibuchi, S.-i. Akiyama, Y. Nishioka
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): T. Kuramoto, H. Goto, H. Uehara, S. Kakiuchi, Y. Maekawa, K. Yasutomo, M. Hanibuchi
Study supervision: H. Goto, S. Kakiuchi, S. Sone, Y. Nishioka

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