Oncogenic fusion gene CD74-NRG1 confers cancer stem cell-like properties in lung cancer through a IGF2 autocrine/paracrine circuit

CD74-NRG1, an oncogenic fusion gene product, leads to insulin-like growth factor 2 autocrine/paracrine circuit and confers cancer stem cell properties

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Abstract

The *CD74-Neuregulin1* (*NRG1*) fusion gene was recently identified in invasive mucinous adenocarcinoma, a malignant type of lung adenocarcinoma, and is considered to be a novel driver gene aberration. However, pathogenic functions of the *CD74-NRG1* fusion gene are unknown, and the mechanism underlying the initiation of cancer stem cells (CSCs) and their maintenance in tumors with oncogenic fusion genes is still unclear. In this study, we observed that expression of the *CD74-NRG1* fusion gene has an activity to increase the population of cells with CSC properties. *CD74-NRG1* expression facilitated sphere formation of not only cancer cells but also non-cancerous lung epithelial cells. Using a limiting dilution assay in a xenograft model, we showed that expression of the *CD74-NRG1* fusion gene enhanced tumor initiation. We observed that *CD74-NRG1* expression stimulates phosphorylation of ErbB2/3 and activates the phosphatidylinositol 3-kinase (PI3K)/Akt/NF-κB signaling pathway. Furthermore, we found that levels of the secreted insulin-like growth factor 2 (IGF2) were increased, and phosphorylation levels of the receptor for IGF2, IGF1 receptor (IGF1R), were enhanced in cells expressing *CD74-NRG1* in an NF-κB activity-dependent manner. These findings suggest that the NF-κB activity stimulated by CD74-NRG1 induces the IGF2 autocrine/paracrine circuit. In addition, tumor sphere formation induced by the *CD74-NRG1* fusion gene was suppressed by inhibitors of ErbB2, PI3K, or NF-κB, or an anti-IGF2 antibody. Our study thus provides a rationale for developing important treatment options to block the signals that contribute to the CSC properties, ErbB/PI3K/Akt/NF-κB pathway, and IGF2 circuit, and to eradicate tumors and prevent their recurrence.

Introduction

Cancer stem cells (CSCs) are thought to be responsible for tumor, recurrence, and drug resistance (1). It is also believed that many cancer cells are actually differentiated cells generated from CSCs, similar to how normal tissues are derived from tissue-specific stem cells (1). By definition, CSCs represent a distinct cell population with self-renewal capacity that can be prospectively isolated. This population of cancer cells was initially identified in acute myeloid leukemia in 1997 (2). Since then, several properties of CSCs have been described, and cancer cells that exhibit some CSC properties have been detected in many solid tumors, including lung cancer and breast cancer (3-6). Because CSCs are thought to be resistant to various stressful conditions such as treatment with chemotherapy and molecular targeted drugs, they may survive regardless of tumor shrinkage. After some time, the small number of therapy-resistant CSCs may start to grow, leading to recurrence associated with drug resistance. Therefore, targeting molecules that play a critical role in maintenance of CSCs is an important

therapeutic strategy to eradicate tumors and prevent recurrence.

Recently, oncogenic fusion genes have been discovered in solid tumors, especially in lung cancer. In lung adenocarcinomas, a major type of lung cancer, oncogene fusions frequently occur and it may act as driver gene aberrations as well as *EGFR* or *KRAS* oncogene mutations. The *ALK*, *RET* and *ROS1* fusion genes have already been reported to be involved in cancer development (7-10). Crizotinib, an inhibitor of ALK kinase, is clinically available and has been shown to be effective in lung adenocarcinomas with *EML4-ALK* fusion. Much effort has been devoted to developing targeted drugs against the tyrosine kinases in the fusion protein for improving therapeutic strategies. However, major concerns regarding recurrence and resistance to the targeted drugs against the tyrosine kinases have emerged, resulting in poor prognosis in cancer patients (11). In fact, it is largely unknown whether the fusion genes are functional in terms of initiation and maintenance of CSCs. If such mechanisms exist, novel therapeutic strategies based on these could be developed.

We and other researchers recently identified the *CD74-Neuregulin1* (*NRG1*) fusion gene in a portion of invasive mucinous adenocarcinomas (IMAs) of the lung (12,13). IMA is a highly malignant type of lung adenocarcinoma that is mainly caused by *KRAS* mutations. However, this fusion gene is found in cancers that lack other targetable oncogene mutations such as *KRAS*, *EGFR*, *BRAF*, and *ERBB2*. Therefore, the fusion gene may play important roles as a driver gene aberration in the development of IMA and has the potential to be a novel therapeutic target. CD74 is a transmembrane protein that consists of extracellular, transmembrane, and intracellular domains. NRG1, also called heregulin, is a ligand for the ErbB3/human epidermal growth factor receptor (HER) 3 tyrosine kinase (14). The CD74-NRG1 protein retains the transmembrane domain of CD74 and the epidermal growth factor (EGF)-like domain of NRG1 (NRG III-β3 form). The NRG III-β3 form protein has a cytosolic N-terminus and a membrane-tethered EGF-like domain, and mediates juxtacrine signaling through ErbB2/ErbB3, because the retained EGF-like domain has biological activity. Following cleavage at the border of the EGF-like domain, NRG1 is also secreted and stimulates ErbB2/ErbB3 in an autocrine/paracrine manner. Recently, other types of fusion genes that include a part of NRG1 have been reported not only in IMA of the lung but also in ovarian cancer (15). The breakpoints in *NRG1* occur in breast cancer tissues (16,17), and the resulting fusion gene products are secreted by a breast cancer cell line (18). Typically, this part of NRG1 is thought to be able to stimulate ErbB2/ErbB3.

We previously reported that the NRG1 protein enhances tumor sphere formation by breast cancer cells by activating signaling pathways through ErbB2/ErbB3 heterodimers (19). The process of sphere formation partly recapitulates the tumorigenic process because only cells with CSC properties are thought to be resistant to anoikis, which is apoptosis caused by the loss of adhesive survival signals in suspension culture, and form clonal spheroids in sphere culture

medium (SCM) which is serum-free but contains several growth factors and hormones (20,21). Similar to breast CSCs, lung CSCs can be grown and isolated using the sphere culture protocol (6,22,23).

In this study, we showed that expression of the CD74-NRG1 fusion protein enhances the efficiency of tumor sphere formation *in vitro* and tumor initiating ability *in vivo*, which are two important criteria for cells with CSC properties (24). The hypothesis that CD74-NRG1 initiates CSCs in lung tissues was further supported by the observation that expression of the fusion gene induced sphere formation by normal lung epithelial cells. We showed that the CD74-NRG1 protein activates the phosphatidyl inositol 3-kinase (PI3K)/Akt/NF-κB signaling pathway. Moreover, the production and secretion of IGF2 protein, which is essential for this tumor sphere formation, was dependent on NF-κB activity. The resulting autocrine/paracrine circuit that involves IGF2 appears to maintain CSCs. To the best of our knowledge, this is the first study reporting that oncogenic fusion gene products indeed function at the level of CSCs. Thus, inhibition of this signaling pathway by targeting a single molecule or several molecules in combination is an efficient way to treat *CD74-NRG1* fusion-positive cancers for eradication of cancer cells.

Materials and Methods

Cell Lines and cell culture

Lung cancer cell line H322 and breast cancer cell line BT20 were purchased from the American Type Culture Collection (ATCC). Cells were cultured in RPMI1640 with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin-streptomycin (P/S; Nacalai). HEK293T cells (ATCC) for lentivirus production were cultured in Dulbecco's Modified Eagle Medium: Nutrient Mixture (DMEM) with 10% FBS and 1% P/S. The cells were maintained in a humidified incubator with 5% CO_2 at 37°C.

Western blot analysis

Immunoblotting was performed using standard procedures as described (19). Anti-ErbB2, p-ErbB2, p-ErbB3, Akt, p-Akt, Nanog, Oct-4, Sox2, IKKα, IKKβ, p-IKKα/β, IκBα, p-IκBα, IGF1R (receptor for IGF2), and p-IGF1R antibodies were purchased from Cell Signaling Technology. Anti-ErbB3 and actin antibodies were purchased from Millipore. Anti-NRG1 and CD74 antibodies were purchased from Thermo Scientific and Abcam, respectively. Proteins were detected with horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies (GE Healthcare Life Sciences).

Sphere formation assay

Sphere formation assay was performed as described (19). Briefly, cells were plated as single cells on ultralow attachment 24-well plates (2000~5000 cells/well). They were grown in sphere culture medium (SCM) which consisted of serum-free DMEM/F-12 medium (GIBCO) supplemented with 20 ng/mL EGF (Millipore), 20 ng/mL basic fibroblast growth factor (bFGF) (PeproTech), B27 (GIBCO) and heparin (Stem Cell Technologies) or in DMEM/F-12 medium with or without inhibitors or antibodies. LY294002 and anti-IGF2 antibody were purchased from Cell Signaling Technology. Lapatinib and Dasatinib were purchased from Selleck Chemicals. DHMEQ was a kind gift from K. Umezawa, Aichi Medical School, Japan. Spheres with a diameter > 75 μm were counted after 4-7 days.

Proliferation assay

Cells were seeded in a 12-well plate at low density (5000 cells/well), cultured in RPMI1640 with 10% FBS and 1% P/S. After 4-6 days, cells were harvested and counted.

Construction of lentiviral vectors for expression of *CD74-NRG1*

Expression vectors were constructed as described previously (12). Briefly, full-length cDNAs were amplified from tumor cDNA by PCR and then inserted into pLenti-6/V5-DEST plasmids (Invitrogen). By using Sanger sequencing, the integrity of inserted cDNA was verified.

Viral infection

H322 cells, BT20 cells, and small airway epithelial cells (SAECs) at 60-70% confluence were infected with empty lentiviruses or *CD74-NRG1*-expressing lentiviruses, and then treated with blasticidin (Invitrogen) (10, 20, and 5 μg/mL, respectively) for stable expression as described previously (12).

Flow cytometry analysis

To identify the breast CSC population, cells were stained with Alexa fluor 647-labeled anti-human CD24 and APC-H7 labeled anti-human CD44 antibodies (BD Pharmingen) at 4 °C for 20 min. Then cells were analyzed with FACSAria II flow cytometer (BD Bioscience). Dead cells were excluded by propidium iodide (PI; Sigma) staining. Data were analyzed with FlowJo software (Treestar).

Quantification of NF-κB activity by Enzyme-Linked ImmunoSorbent Assay (ELISA)

Nuclear extracts were prepared with a Nuclear Extract kit (Active Motif), and NF-κB subunit p65-DNA binding activity was measured with a TransAM NF-κB p65 Transcription Factor

Assay kit (Active Motif). All procedures were performed according to the manufacturer's protocol.

Measurement of IGF2 concentration in culture medium

Cells were seeded in 60 mm dishes and cultured in RPMI medium with 10% FBS. At 60-70% confluence, the medium was changed to 1.5 mL of RPMI without FBS. After 24 hr incubation, the medium was collected for IGF2 measurement assay. We concentrated 500 μL culture medium to 50 μL by using a microcon (Millipore) and then measured the IGF2 concentration with the IGF2 Human ELISA kit (Mediagnost). All procedures were performed according to the manufacturer's protocol.

Xenografts

Cells were admixed with 50 μl Matrigel (BD Biosciences) and the cell mixture was injected into the right flank of 8-week-old nude mice. Tumors larger than 200 mm^3 were counted. Tumor volume was measured 2 times a week using the following formula: $V=1/2(LxW^2)$, where L equals length, and W equals width.

Statistical analysis

All data are presented as the mean ± SE. The unpaired Student *t*-test was used to compare differences between two samples and values of $p < 0.01$-0.05 (*), $p < 0.001$-0.01 (**) or $p < 0.001$(***) were considered significant. Tumor initiating frequency was calculated using the ELDA Software (25).

Study approval

Mice were handled according to the guidelines of National Cancer Center Research Institute, Institute of Medical Science, the University of Tokyo and Kanazawa University. The experiments were approved by the committees for animal research at National Cancer Center Research Institute, Institute of Medical Science, the University of Tokyo and Kanazawa University.

Results

・CD74-NRG1 protein induces sphere formation of cancer cells.

We first examined whether the CD74-NRG1 fusion protein induces sphere formation by lung cancer cells. To evaluate the sphere forming ability of *CD74-NRG1*-expressing cells, we infected lentivirus encoding cDNA for the *CD74-NRG1* fusion gene, C6;N6 and C8;N6 variants,

as reported by Nakaoku et al. (12), into H322 lung cancer cells (Fig. 1A and B). These two variants are different in the breakpoints of *CD74*. We chose H322 cells for this study because they have no *KRAS* mutations. When we cultured these cells in conventional SCM containing EGF, bFGF, and B27 supplement, they generated spheres with similar efficiency as cells infected with lentivirus carrying a control vector (Fig. 1C and D). Intriguingly, *CD74-NRG1*-expressing cells also generated spheres when cultured in medium without EGF, bFGF, or B27 supplement, whereas control cells did not (Fig. 1C and D). Because CSC-related function of NRG1 protein was originally identified in breast cancer cells (19), we constructed *CD74-NRG1*-expressing BT20 breast cancer cells to investigate the mammosphere forming ability (Fig. 1B). *CD74-NRG1*-expressing breast cancer cells formed mammospheres even when cultured in a medium without EGF, bFGF, or B27 supplement (Fig. 1E and F). These findings indicate that the CD74-NRG1 fusion protein induces tumor sphere forming ability in lung and breast cancer cells.

・The CSC population increased in *CD74-NRG1*-expressing cells.

Next we examined expression levels of the stem cell marker proteins, Nanog, Oct-3/4 and Sox2 (26). These stem cell markers were expressed at higher levels in *CD74-NRG1*-expressing H322 cells than in control cells (Fig. 2A). Also, in *CD74-NRG1*-expressing BT20 cells, expression levels of the stem cell markers were higher than in control cells (Fig. 2B). In breast cancer, the $CD44^{high}/CD24^{-/low}$ cell population is enriched with cancer cells with stem-like properties (3,27). When we investigated the proportion of $CD44^{high}/CD24^{-/low}$ CSC-enriched cells by flow cytometry, the percentages of the $CD44^{high}/CD24^{-/low}$ population increased from 1.94% to 9.47% (C6;N6 variant) or 8.21% (C8;N6 variant) (Fig. 2C). These data further support the idea that the *CD74-NRG1* fusion gene can enhance CSC properties. On the other hand, the proliferation assay revealed that the CD74-NRG1 protein did not significantly induce cell growth in adherent cultures (Fig. 2D and E) in the medium containing 10 % FBS or the starvation medium with 0.5 % FBS (Fig. 2F).

・The CD74-NRG1 protein activates the PI3K/Akt pathway and controls sphere formation ability.

Because the CD74-NRG1 fusion protein contains the functional domain of NRG1, we hypothesized that activation of the ErbB2/ErbB3-regulated pathway contributes to sphere formation by *CD74-NRG1*-expressing cells. To test this hypothesis, we examined the phosphorylation levels of ErbB2, ErbB3, and Akt. Expression of the CD74-NRG1 fusion protein increased the phosphorylation levels of ErbB2, ErbB3, and Akt compared with vector control cells (Fig. 3A). Similar results were observed in *CD74-NRG1*-expressing BT20 cells

(Fig. 3B). These data show that the CD74-NRG1 fusion protein activates ErbB2 and ErbB3 heterodimer receptors, leading to PI3K/Akt pathway activation. Then, to investigate whether activation of the ErbB signaling pathway is important for tumor sphere formation, we checked the effect of Lapatinib, an ErbB2 tyrosine kinase inhibitor, on the formation of tumor spheres. Lapatinib significantly suppressed the sphere forming ability of *CD74-NRG1*-expressing H322 cells (Fig. 3C). This result indicates that signals through ErbB2/ErbB3 receptors play important roles in generating tumor spheres by *CD74-NRG1*-expressing cells. Furthermore, Lapatinib but not Dasatinib, a Bcr-Abl tyrosine kinase inhibitor, inhibited tumor sphere formation by *CD74-NRG1*-expressing BT20 cells (Fig. 3D).

・NF-κB activation contributes to sphere formation by *CD74-NRG1*-expressing cells.

The NF-κB transcription factor complex, a downstream target of Akt, is activated by the NRG-stimulated ErbB2/ErbB3 signaling pathway (19). We then investigated whether NF-κB signaling is activated in *CD74-NRG*-expressing cells. The NF-κB transcription factor complex is usually inactive and bound to IκBα, an inhibitory protein, in the cytoplasm (28). IKKα/β are the upstream kinases involved in the phosphorylation of IκBα, which results in its ubiquitination, proteasome-mediated degradation and the subsequent release of NF-κB. The released NF-κB translocates to the nucleus and binds to the κB sequence, where it promotes the transcription of various genes. We compared phosphorylation levels of these proteins in *CD74-NRG1*-expressing cells and control cells. In *CD74-NRG1*-expressing cells, phosphorylation levels of IKKα/β and IκBα were increased (Fig. 4A). To examine the DNA-binding activity of NF-κB subunit p65 in *CD74-NRG1*-expressing cells, we quantified the intensity of the p65/DNA complex formation by ELISA. Expression of CD74-NRG1 fusion protein led to a marked increase in the DNA-binding activity of p65 (Fig. 4B and C). Thus the CD74-NRG1 protein-stimulated ErbB2/ErbB3 signaling pathway appears to activate PI3K/Akt, leading to NF-κB activation. To test whether activation of PI3K or NF-κB is involved in the sphere formation ability of *CD74-NRG1*-expressing cells, we treated these cells with LY294002 and DHMEQ, specific inhibitors of PI3K and NF-κB, respectively (29). LY294002 or DHMEQ suppressed sphere formation at the similar levels in both H322 and BT20 cells (Fig. 4D and E). These data indicate that PI3K/Akt/NF-κB pathway induces tumor sphere forming ability.

・IGF2 plays important roles in sphere formation induced by the CD74-NRG1 fusion protein.

We have recently found that IGF2 is a downstream target of NF-κB upon stimulation with NRG (Tominaga K, Murayama T, et al. unpublished). We next measured secreted IGF2 protein in culture medium. The amount of IGF2 protein was increased by *CD74-NRG1* expression: 0.91

ng/mL and 0.98 ng/mL in cells expressing C6;N6 and C8;N6 variants of CD74-NRG1, respectively, compared with 0.71 ng/mL in control cells (n = 2). To investigate whether secreted IGF2 is involved in sphere formation, we added IGF2 neutralizing antibody to the medium and measured sphere forming efficiency in H322 cells and BT20 cells. The IGF2 neutralizing antibody greatly decreased sphere forming efficiency in cells expressing either variant of CD74-NGR1 but not in control cells (Fig. 5A, B). In order to check whether IGF1R, a receptor for IGF2, is activated by secreted IGF2 that is induced by CD74-NRG1-NF-κB pathway, we treated H322 cells expressing C8;N6 variants of CD74-NRG1 with or without DHMEQ and examined phosphorylation of IGF1R. We found that the phosphorylation levels of IGF1R were increased by expression of CD74-NRG1 protein (Fig. 5C). The increased phosphorylation levels of IGF1R were reduced by DHMEQ treatment. These results suggest that the CD74-NRG1 protein induces sphere formation by activating the PI3K/Akt/NF-κB/IGF2 signaling pathway and the IGF2 autocrine/paracrine circuit.

・The *CD74-NRG1* fusion gene enhances the tumor initiating ability.

We next examined whether *CD74-NRG1* expression alters the tumor initiating ability using a limiting dilution assay in a xenograft model. We injected 1×10^2, 1×10^3, 1×10^4, or 1×10^5 cells subcutaneously into the right flank of nude mice and observed tumorigenesis. *CD74-NRG1*-expressing H322 cells induced tumor formation more efficiently than control cells (Table). However, the tumor growth rate was not significantly increased by *CD74-NRG1* expression (Fig. 6A). These results indicate that the CD74-NRG1 fusion protein has tumor initiating ability *in vivo*.

・The CD74-NRG1 protein induces sphere formation of normal lung epithelial cells.

Finally, we extended our analysis to non-cancerous cells. Using the lentivirus system, we created *CD74-NRG1*-expressing SAECs, which are immortalized normal lung epithelial cells (30). We analyzed the efficiency of sphere formation by these cells in serum-free medium without EGF, bFGF or B27 supplement. We found that *CD74-NRG1*-expressing SAECs formed spheres, but control SAECs formed no spheres. These results support the notion that CD74-NRG1 initiates CSCs in lung tissues without requiring paracrine factors such as growth factors, indicating that this fusion gene is a strong driver gene (Fig. 6B and C).

Discussion

In this study, we provide evidence that the *CD74-NRG1* fusion gene appears to play critical roles in the initiation and maintenance of CSC properties. Moreover, we clarified the signaling

pathways controlled by the CD74-NRG1 protein. The CD74-NRG1 protein activates the ErbB/PI3K/NF-κB pathway, which leads to activation of the IGF2-autocrine/paracrine circuit. Thus, oncogenesis may occur at the level of CSCs. It is reasonable to hypothesize that the CD74-NRG1 protein confers CSC properties on a few immature, progenitor-like cells rather than on the terminally differentiated cancer cells. This finding is important, because therapy targeting a single molecule or several molecules in combination in this pathway may eradicate tumors and prevent recurrence (Fig. 6D).

IMAs of the lung constitute 2%−10% of all lung adenocarcinomas in Japan and the United States, and are regarded as more malignant than other more common types of lung adenocarcinomas (31-33). The *KRAS* mutation was the only driver aberration found in IMAs. However, to improve clinical outcomes, it is necessary to identify novel driver aberrations in *KRAS*-negative IMAs. Following much effort, the *CD74-NRG1* fusion gene was identified in IMAs in 2014 (12,13). This fusion gene is mutually exclusive with *KRAS* mutations. In *CD74-NRG1* fusion-positive tumors, enhanced expression of *NRG1* is observed (13). *CD74-NRG1* is regarded as a driver gene aberration in the development of IMA. Since it is recently reported that the fusion genes that include a part of *NRG1* are found in ovarian cancer (15), the gene alteration involving *NRG1* fusion may also occur in other types of tumors.

In breast cancer cells and tissues, breaks within *NRG1* are frequently detected and may be responsible for *NRG1* gene fusion (16,17). In fact, *DOC4-NRG1* fusion gene is observed in the MDA-MB-175 breast cancer cell line (18). In this cell line, NRG1 expression is enhanced because of the promoter activity of *DOC4* and NRG1 is produced in the culture medium. It is, thus, reasonable that some breast cancers are caused by *NRG1* gene fusion in a manner similar to that observed in *CD74-NRG1* fusion-positive IMAs. We are currently trying to identify important gene aberrations including gene fusions in breast cancer by using next-generation sequencing.

In this study, we showed that expression of the CD74-NRG1 protein not only induces sphere forming ability *in vitro* but also enhances the tumor-initiating ability *in vivo*. These findings indicate that this fusion gene is involved in tumor development by inducing CSC properties and will be an effective therapeutic target for these tumors. Moreover, we showed that the CD74-NRG1 protein activates the PI3K/Akt/NF-κB signaling pathway, leading to IGF2 autocrine/paracrine circuit to initiate and maintain cells with CSC properties. Our findings provide a rationale for developing alternative treatment options, despite the emergence of acquired resistance. Because the major acquired resistance mechanisms have been reported to be additional mutations in the tyrosine kinase domain of the fusion gene, effective drugs targeting the mutant tyrosine kinases have been developed (34). Our findings suggest that targeting other molecules in this pathway, rather than the mutant tyrosine kinases themselves,

for the initiation and maintenance of CSCs, may be equally effective.

It does not seem that expression of the CD74-NRG1 protein strongly stimulates cell growth *in vitro* and *in vivo*. It is thought that cells with CSC properties grow rather slower than other differentiated cancer cells (1). It is thus reasonable that the CSC properties conferred by expression of the CD74-NRG1 are not strongly associated with stimulation of cell growth. Since the important characteristics of CSCs are resistance to stressful conditions, the CD74-NRG1-expressing cancer cells may be more resistant to conventional chemotherapeutics than those without expression of the CD74-NRG1 protein.

Expression of CD74-NRG1 increased the percentage of $CD44^{high}/CD24^{-/low}$ CSC-enriched cells from 1.94% to ~9.5%. The fact that $CD44^{high}/CD24^{-/low}$ CSC-enriched cells are still a minor population indicates that expression of the CD74-NRG1 protein is not sufficient for conferring CSC properties on all cells. Since the parental BT20 cells do not express the CD74-NRG1 gene, it is possible that the intrinsic CSC properties carried by a subpopulation of BT20 cells are conferred by other gene alterations. However, the increase in the population of BT20 cells with CSC properties by enforced CD74-NRG1 expression may indicate that the intrinsic CSC properties are conferred on more cells by the IGF2 autocrine/paracrine circuit. It is known that even within a cancer cell line, there are immature cell populations with CSC properties and other differentiated cancer cell populations (27, 35). It is thus possible that expression of the CD74-NRG1 protein shifts the cell population toward more cells with CSC properties than differentiated cells.

The mechanisms underlying the production of IGF2 by NF-κB are unclear. We recently found that NRG stimulates the transcription of *IGF2* mRNA in an NF-κB-dependent manner (Tominaga K, Murayama T, et al. unpublished). Several binding site motifs for NF-κB are present in the *IGF2* promoter sequence. Thus, increased NF-κB activity may lead to production of IGF2 at the transcriptional level.

In conclusion, our results suggest that PI3K/Akt/NF-κB/IGF2 signaling activated by the CD74-NRG1 fusion protein is involved in CSC maintenance and tumor initiation. Therefore, development of efficient inhibitors or antibodies targeting the molecules in this pathway is anticipated to improve the prognosis of IMA patients with the *CD74-NRG1* fusion gene. Further, establishment of effective diagnostic methods capable of detecting this gene aberration is necessary. A therapeutic strategy that targets cells with CSC properties by inhibiting PI3K/Akt/NF-κB may be useful in other types of cancers caused by *NRG1* gene fusions, besides IMAs.

Author's Contributions

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Table

Results of limiting dilution assay of vector- or CD74-NRG1-transduced H322 cells

	H322 cells					
	Cells (per site)				Tumor initiating cell frequency estimate	Probability
	10^2	10^3	10^4	10^5		
Empty vector	1/6	3/6	6/6	5/6	13,101	5.27E-06
CD74-NRG1 (C6;N6)	4/6	5/6	5/6	6/6	1,507	

Figure legends

Figure 1. The CD74-NRG1 fusion protein induces sphere formation.

A, schematic representations of wild-type proteins and the CD74-NRG1 fusion protein (C6;N6 variant: top and C8;N6 variant: bottom). TM, transmembrane domain, Blue arrows indicate the breakpoints. B, expression of the CD74-NRG1 fusion protein in H322 (left) and BT20 (right) cells were confirmed with immunoblot analysis. C and D, sphere formation assay with vector- or CD74-NRG1-transduced H322 cells. Cells were treated with no growth factors or EGF/bFGF/B27 (N.T.: not treated, n = 4, ***P < 0.001). Scale bar = 100 μm. E and F, sphere formation assay with vector- or CD74-NRG1-transduced BT20 cells. Cells were treated with no growth factors or EGF/bFGF/B27 (N.T.: not treated, n = 4, ***P < 0.001). Scale bar = 100 μm.

Figure 2. The CD74-NRG1 fusion protein increases the proportion of CSCs.

A, expression levels of Nanog, Oct3/4 and Sox2 in H322 cells with each vector were determined by immunoblotting. B, expression levels of Nanog, Oct3/4 and Sox2 in BT20 cells with each vector were determined by immunoblotting. C, vector- or CD74-NRG1-trasduced BT20 cells were stained with CD44 and CD24 antibodies and then subjected to flow cytometry analysis. D and E, H322 cells (D) or BT20 cells (E) with each vector were seeded in a 12-well plate (5,000 cells/well). Cells were cultured in 10 % FBS and then harvested and counted after 4 or 6 days (n = 4). F, H322 cells were seeded described in (D). Cells were starved in 0.5 % FBS and then harvested and counted after 6 days (n = 4).

Figure 3. Activation of the PI3K/Akt pathway induces sphere formation.

A and B, phosphorylation levels of ErbB2, ErbB3 and Akt in vector- or CD74-NRG1-transduced H322 cells (A) and BT20 cells (B) were determined by immunoblotting. C and D, sphere formation assay with vector- or CD74-NRG1-transduced H322 cells (C) or BT20 cells (D). Cells were incubated with or without 100 nM Lapatinib or 100 nM Dasatinib (N.T.: not treated, n = 4, ***P < 0.001).

Figure 4. NF-κB is activated by CD74-NRG1 expression.

A, phosphorylation levels of IKKα/β and IκBα in vector- or CD74-NRG1-transduced H322 cells were determined by immunoblotting. B and C, the DNA binding activities of p65 in H322 (B) and BT20 (C) cells were quantified by ELISA (n = 3, *P < 0.05, **P < 0.01) D and E, sphere formation assay with vector- or CD74-NRG1-transduced H322 (D) and BT20 (E) cells. Cells were incubated with or without 1 μg/mL DHMEQ or 1 μM LY294002 (N.T.: not treated, n = 4, ***P < 0.001).

Figure 5. Secreted IGF2 is crucial for sphere formation.

A and B, sphere formation assay with vector- or CD74-NRG1-transduced H322 (A) and BT20 (B) cells. Cells were incubated with 10 μg/mL anti-IGF2 neutralizing antibody (NAb) (N.T.: not treated, data are the mean ± SE; n = 4, ***P < 0.001). C, H322 cells expressing C8;N6 were incubated with or without 5 μg/mL DHMEQ for 24hr. Phosphorylation levels of IGF1R were determined by immunoblotting.

Figure 6. The CD74-NRG1 fusion protein enhances the tumor initiating ability of cancer cells.

A, tumor growth curves of vector- or CD74-NRG1-transduced H322 cells when injected with 1 × 10^4 cells (n = 6). B and C, sphere formation assay with vector- or CD74-NRG1-transduced SAECs. (N.T.: not treated, n = 4, ***P < 0.001). Scale bar = 100 μm. D, The CSC maintenance signals. The CD74-NRG1 protein activates PI3K/Akt/NF-κB pathway to induce IGF2 autocrine/paracrine circuit for maintenance of CSCs. Blocking one or several molecules in the pathways would be effective for eradicating tumors.

Figure 1

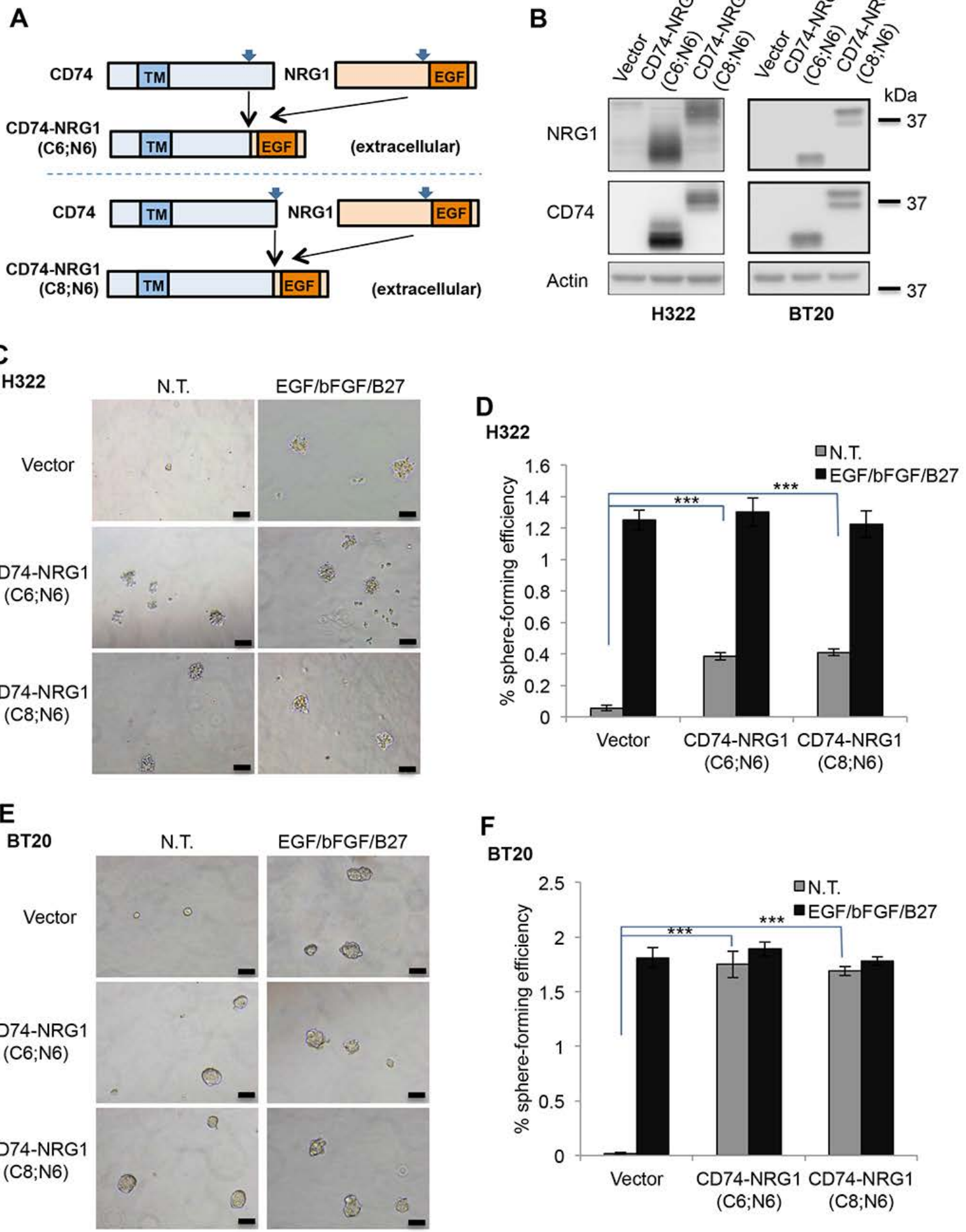
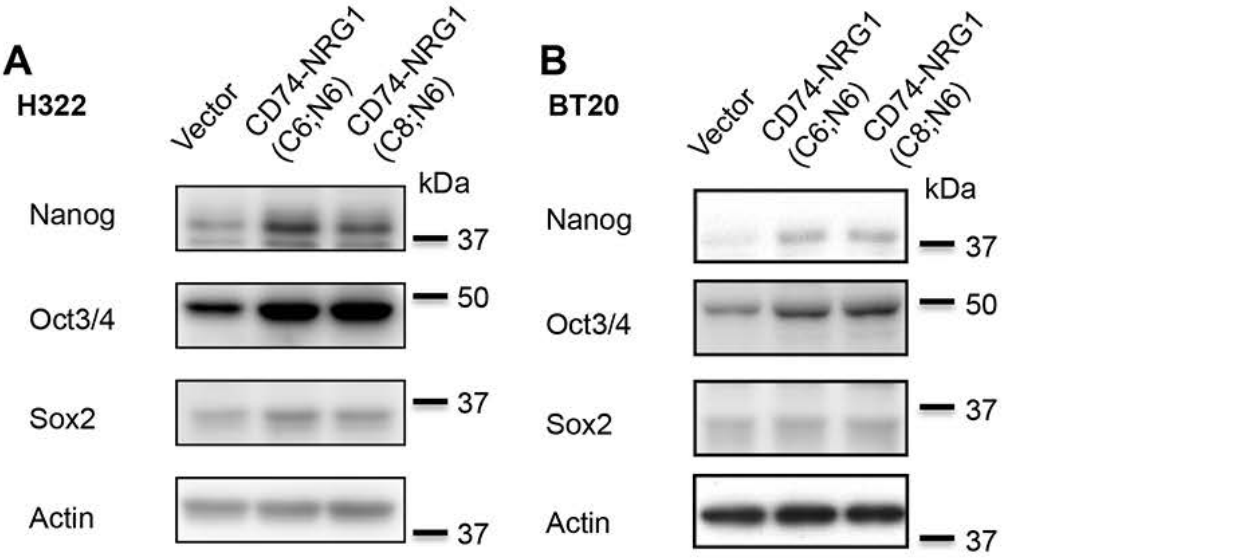
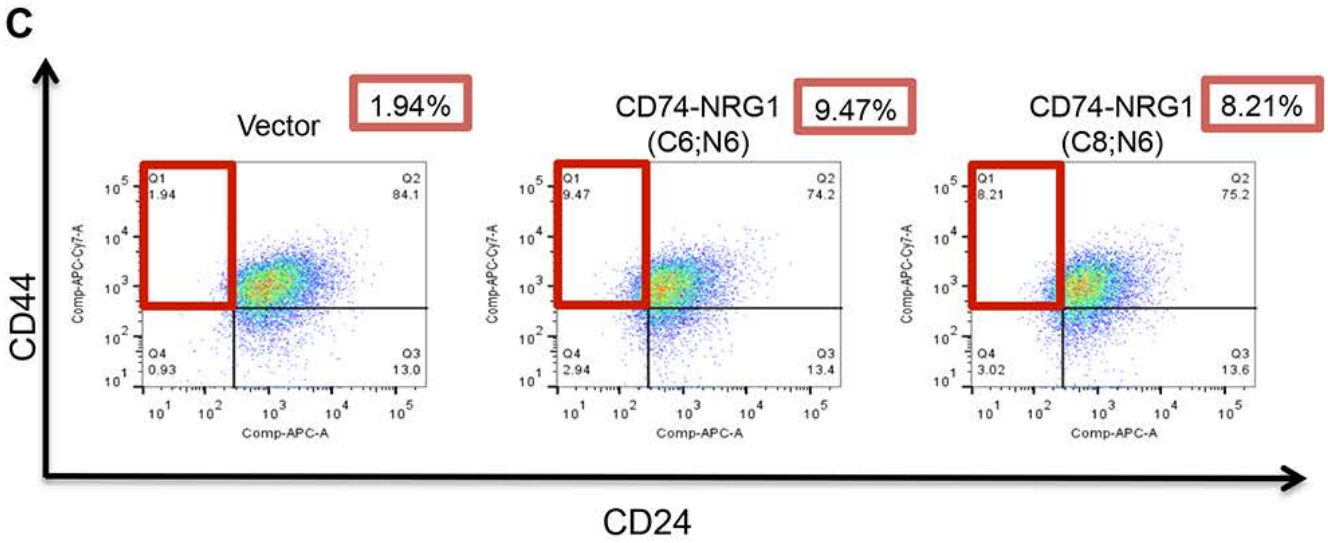
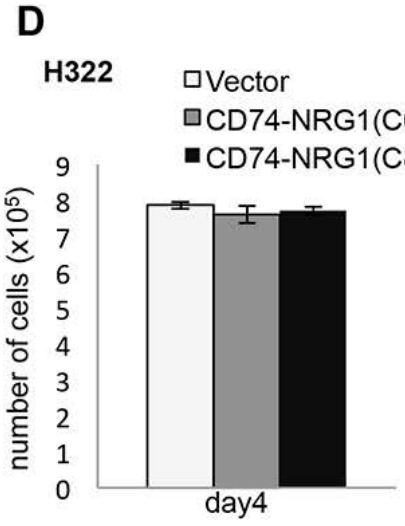
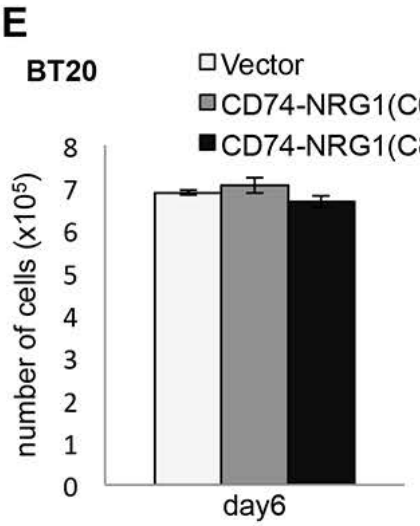

A
CD74
TM
NRG1
EGF
CD74-NRG1
(C6;N6)
(extracellular)
CD74-NRG1
(C8;N6)
B
Vector
CD74-NRG1 (C6;N6)
CD74-NRG1 (C8;N6)
kDa
NRG1
CD74
Actin
37
H322
BT20
C
H322
N.T.
EGF/bFGF/B27
Vector
CD74-NRG1 (C6;N6)
CD74-NRG1 (C8;N6)
D
H322
% sphere-forming efficiency
***
E
BT20
F
BT20

Figure 2


A
H322
Vector
CD74-NRG1 (C6;N6)
CD74-NRG1 (C8;N6)
kDa
Nanog
37
Oct3/4
50
Sox2
37
Actin
37
B
BT20
Vector
CD74-NRG1 (C6;N6)
CD74-NRG1 (C8;N6)
kDa
Nanog
37
Oct3/4
50
Sox2
37
Actin
37


C
Vector
1.94%
CD74-NRG1 (C6;N6)
9.47%
CD74-NRG1 (C8;N6)
8.21%
CD44
CD24
Comp-APC-Cy7-A
Comp-APC-A
Q1 1.94
Q2 84.1
Q4 0.93
Q3 13.0
Q1 9.47
Q2 74.2
Q4 2.94
Q3 13.4
Q1 8.21
Q2 75.2
Q4 3.02
Q3 13.6


D
H322
Vector
CD74-NRG1(C6;N6)
CD74-NRG1(C8;N6)
number of cells (x10^5)
day4


E
BT20
Vector
CD74-NRG1(C6;N6)
CD74-NRG1(C8;N6)
number of cells (x10^5)
day6

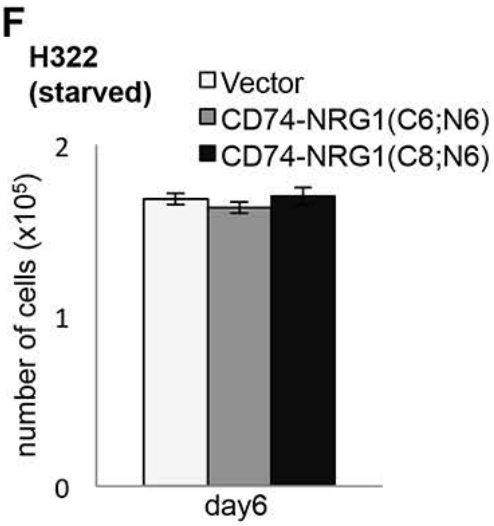
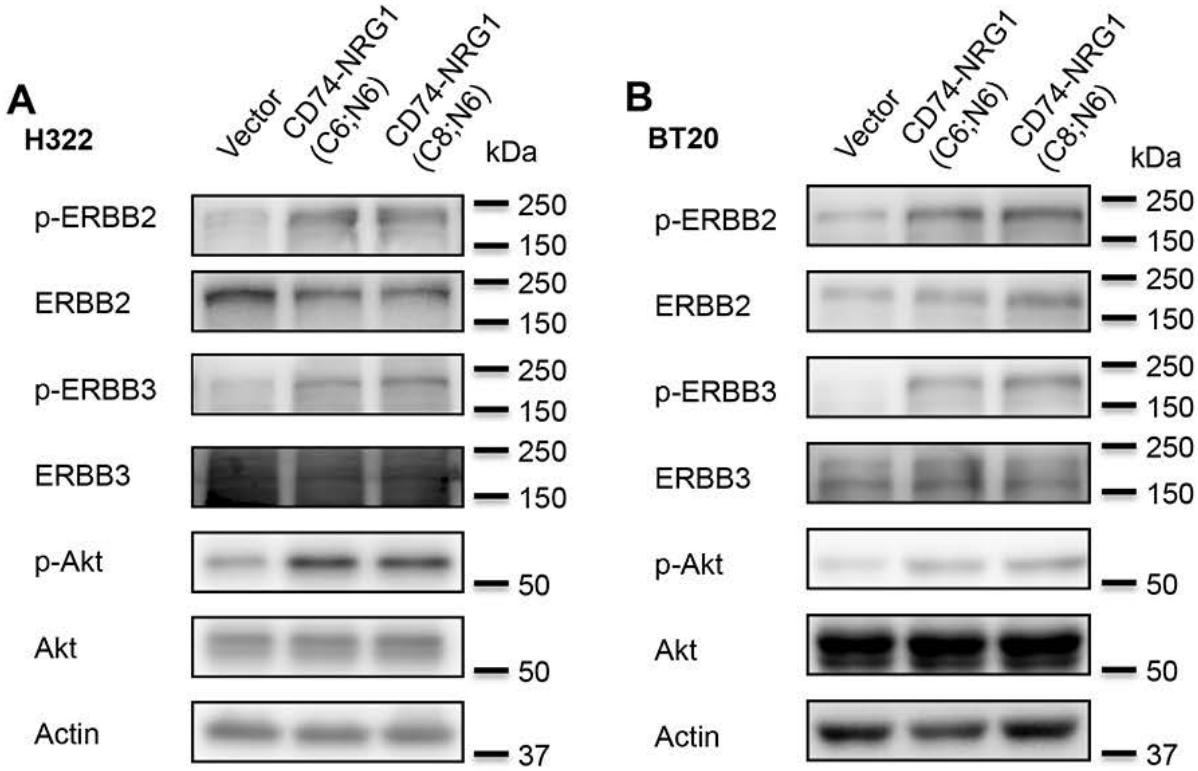
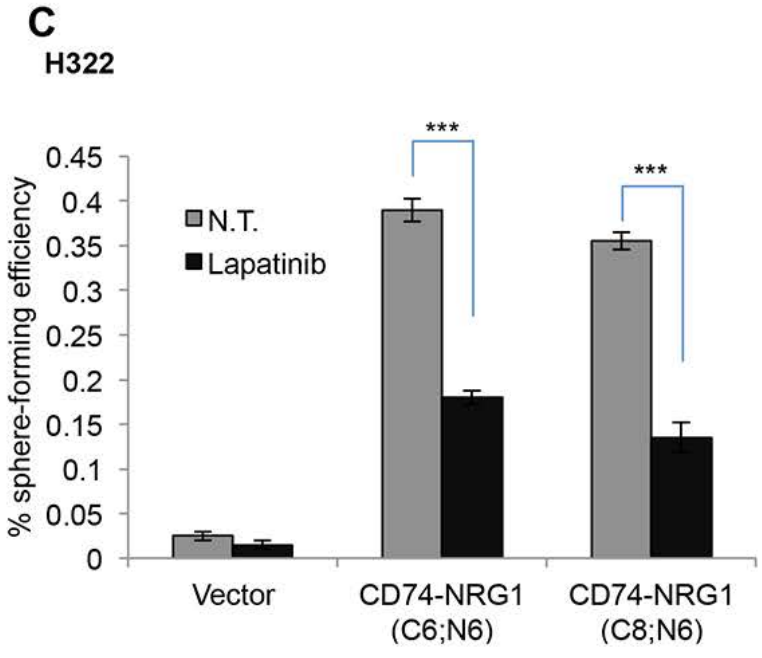

F
H322 (starved)
Vector
CD74-NRG1(C6;N6)
CD74-NRG1(C8;N6)
number of cells (x10^5)
day6

Figure 3


A
H322
Vector
CD74-NRG1 (C6;N6)
CD74-NRG1 (C8;N6)
kDa
p-ERBB2
ERBB2
p-ERBB3
ERBB3
p-Akt
Akt
Actin
250
150
50
37
B
BT20


C
H322
% sphere-forming efficiency
N.T.
Lapatinib
***
Vector
CD74-NRG1 (C6;N6)
CD74-NRG1 (C8;N6)

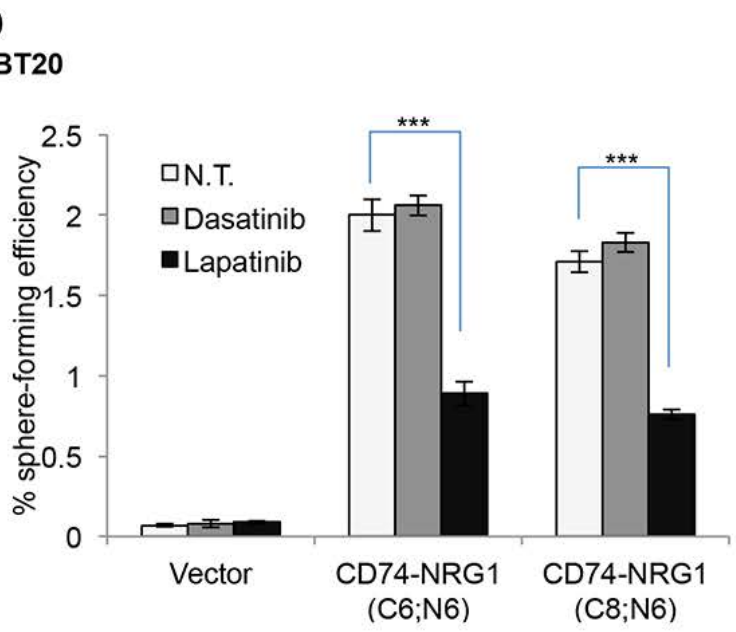
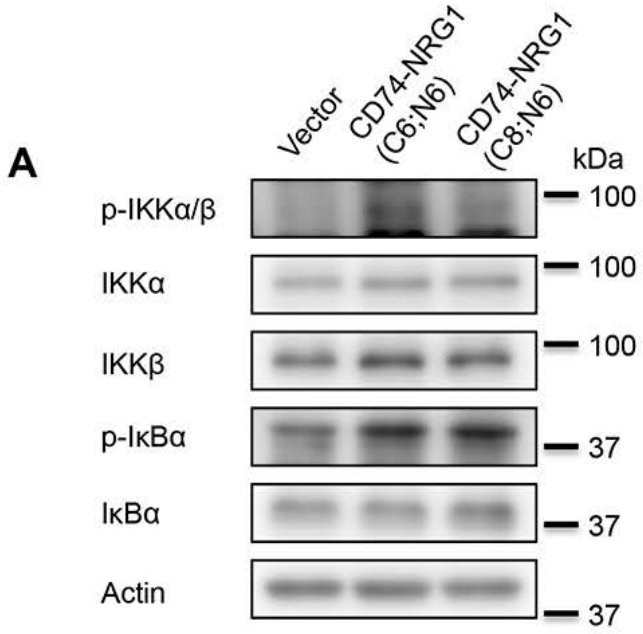
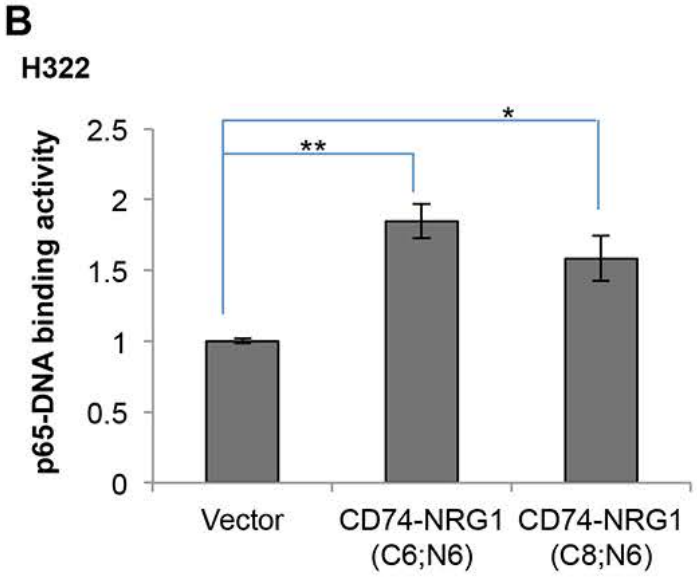
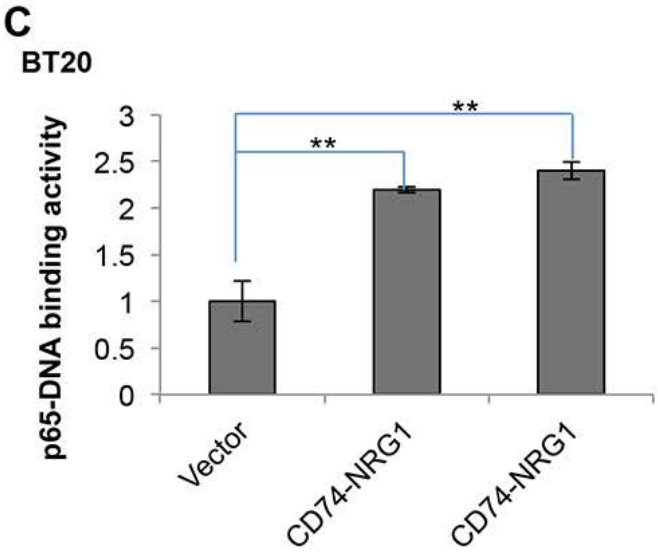
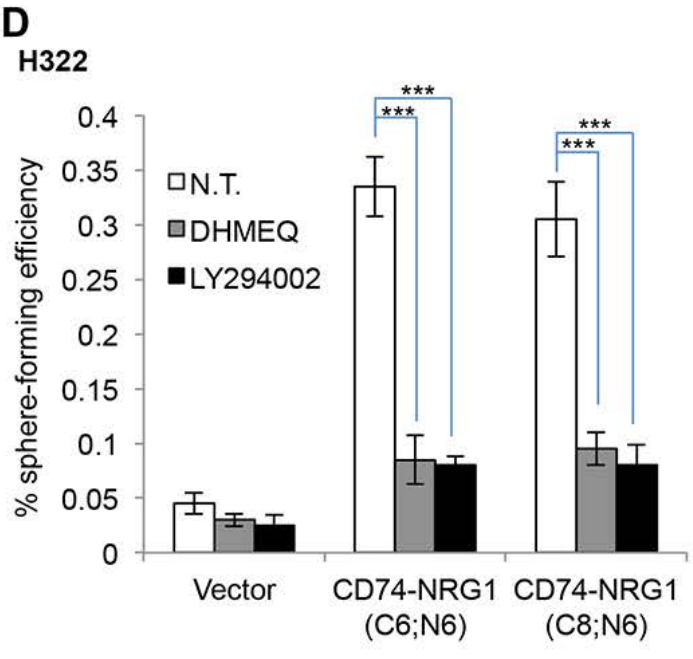

D
BT20
% sphere-forming efficiency
N.T.
Dasatinib
Lapatinib
***
Vector
CD74-NRG1 (C6;N6)
CD74-NRG1 (C8;N6)

Figure 4


A
Vector
CD74-NRG1 (C6;N6)
CD74-NRG1 (C8;N6)
kDa
p-IKKα/β
IKKα
IKKβ
p-IκBα
IκBα
Actin
100
100
100
37
37
37


B
H322
p65-DNA binding activity
2.5
2
1.5
1
0.5
0
*
**
Vector
CD74-NRG1 (C6;N6)
CD74-NRG1 (C8;N6)


C
BT20
p65-DNA binding activity
3
2.5
2
1.5
1
0.5
0
**
**
Vector
CD74-NRG1
CD74-NRG1


D
H322
% sphere-forming efficiency
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
N.T.
DHMEQ
LY294002
***
***
***
***
Vector
CD74-NRG1 (C6;N6)
CD74-NRG1 (C8;N6)

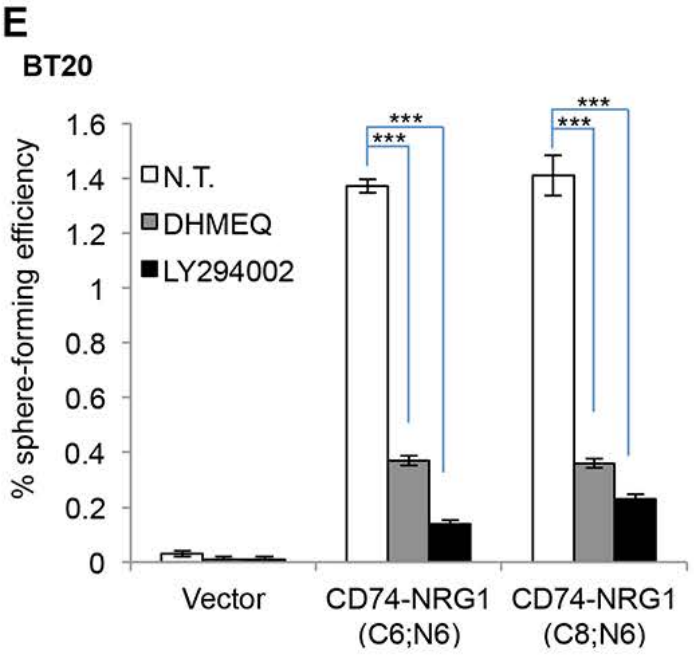

E
BT20
% sphere-forming efficiency
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
N.T.
DHMEQ
LY294002
***
***
***
***
Vector
CD74-NRG1 (C6;N6)
CD74-NRG1 (C8;N6)

Figure 5

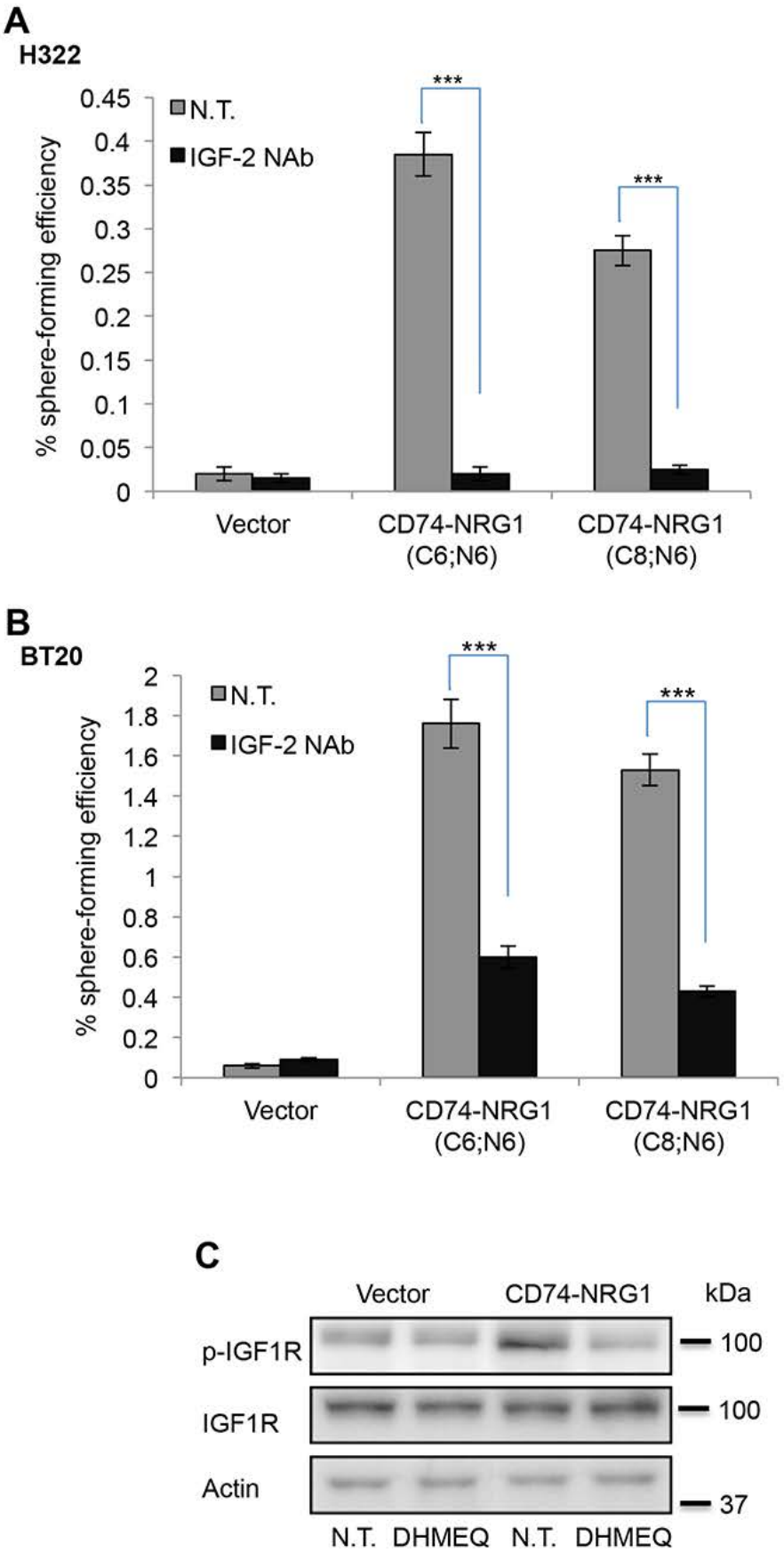
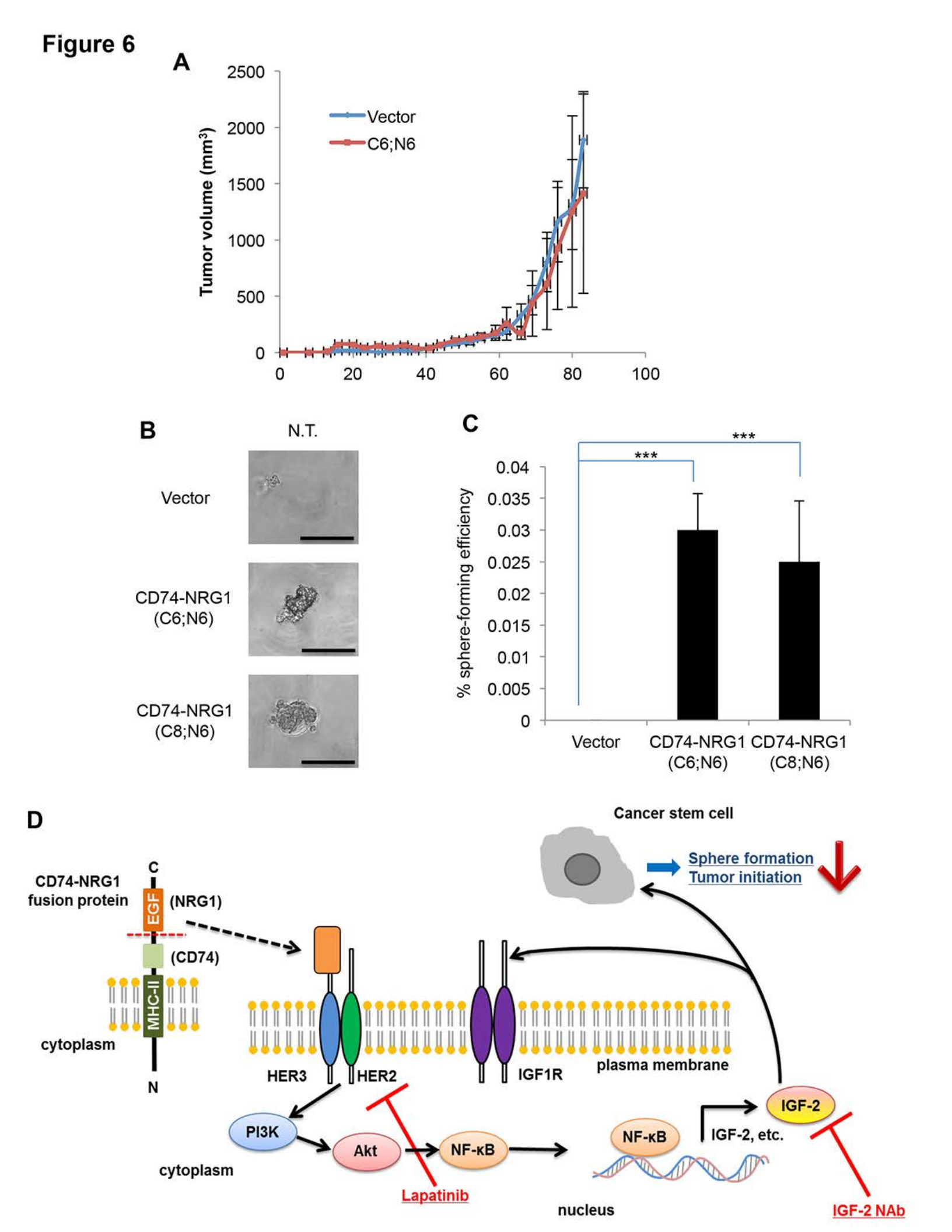

A
H322
% sphere-forming efficiency
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
N.T.
IGF-2 NAb
***
***
Vector
CD74-NRG1 (C6;N6)
CD74-NRG1 (C8;N6)
B
BT20
% sphere-forming efficiency
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
N.T.
IGF-2 NAb
***
***
Vector
CD74-NRG1 (C6;N6)
CD74-NRG1 (C8;N6)
C
Vector
CD74-NRG1
kDa
p-IGF1R
100
IGF1R
100
Actin
37
N.T.
DHMEQ
N.T.
DHMEQ

Figure 6


A
Tumor volume (mm³)
2500
2000
1500
1000
500
0
0
20
40
60
80
100
Vector
C6;N6
B
N.T.
Vector
CD74-NRG1
(C6;N6)
CD74-NRG1
(C8;N6)
C
% sphere-forming efficiency
0.04
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0
***
***
Vector
CD74-NRG1
(C6;N6)
CD74-NRG1
(C8;N6)
D
Cancer stem cell
Sphere formation
Tumor initiation
CD74-NRG1
fusion protein
C
EGF
(NRG1)
(CD74)
MHC-II
cytoplasm
N
HER3
HER2
IGF1R
plasma membrane
PI3K
Akt
NF-κB
cytoplasm
Lapatinib
NF-κB
IGF-2
IGF-2, etc.
nucleus
IGF-2 NAb