

ORIGINAL ARTICLE

ErbB4-mediated regulation of vasculogenic mimicry capability in breast cancer cells

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Abstract

ErbB4 is a member of the ErbB receptor tyrosine kinase family. It has both pro- and anti-oncogenic activities in tumors. Vasculogenic mimicry (VM), a phenomenon in which cancer cells form capillary-like structures without endothelial cells, has been recognized to be a cause of malignant phenotypes in some solid tumors. Here, we used an in vitro VM formation assay, and demonstrated that ErbB4 negatively regulated VM formation in human breast cancer cells. By using CRISPR/Cas9-mediated gene knockout, we verified that the depletion of endogenous ErbB4 improved the VM formation capability. Although treatment with neuregulin 1 (NRG1), a ligand of ErbB4, induced the phosphorylation of ErbB4 and promoted VM formation in a dose-dependent manner, it did not induce such activities in kinase-dead K751M ErbB4-overexpressing cells. Moreover, we examined the effect of the missense mutation E872K of ErbB4, which has been reported in multiple tumors, on VM formation, and found that the mutation enhanced the basal phosphorylation level and ErbB4-mediated VM formation in the absence of NRG1 stimulation. Whereas NRG1 stimulated VM formation, excessive activation of ErbB4 induced a negative effect. In E872K ErbB4-overexpressing cells, but not in wild-type ErbB4-overexpressing cells, the number of VM tubes was significantly decreased by low-dose treatment with the ErbB inhibitor afatinib. Taken together, our findings demonstrated the significance of ErbB4-mediated VM formation, and suggested the possibility of *ErbB4* mutations as effective targets in breast cancer.

KEYWORDS

afatinib, ErbB4, neuregulin, receptor tyrosine kinase, vasculogenic mimicry

1 | INTRODUCTION

The ErbB receptors (EGFR, HER2, ErbB3, and ErbB4) are RTKs that regulate various cell properties through intracellular signaling

pathways, and the overexpression of ErbB receptors is associated with tumorigenesis.¹⁻³ Small molecule inhibitors and monoclonal antibodies that target EGFR and HER2 have been developed as anti-tumor drugs, and have been successfully used in cancer patients.⁴

Abbreviations: CRISPR, clustered regularly interspaced palindromic repeat; CYT, cytoplasmic; ICD, intracellular domain; JM, juxtamembrane; NRG1, neuregulin 1; RTKs, receptor tyrosine kinases; VEGFR, vascular endothelial growth factor receptor; VM, vasculogenic mimicry.

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ErbB3 is also recognized as an oncogene, and an anti-ErbB3 monoclonal antibody was recently tested in clinical trials.⁵ Although the activation of EGFR, HER2, and ErbB3 is associated with the malignant phenotypes of various cancer types, the functions of ErbB4 in tumors appear to be more complex, and they remain controversial.

In 1993, ErbB4 was identified as a receptor that is activated by specific ligands.⁶ Unlike other ErbB receptors, several studies have indicated that the expression of ErbB4 protein is associated with a favorable prognosis in breast cancer patients.^{7,8} Mechanistically, the ErbB4 intracellular domain (ICD) is proteolytically released and translocated to the mitochondria after ligand stimulation to induce apoptosis.^{9,10} Conversely, ErbB4 activates oncogenic signaling pathways, such as PI3K/Akt and MAPK pathways,^{9,11} and some tumors exhibit an increased expression level of ErbB4.¹²

Many studies have shown that RTKs frequently mutate in cancers, thereby inducing constitutive activation or drug resistance.¹³⁻¹⁵ Several comprehensive analyses have revealed gene alterations of *ErbB4* in tumors, including breast cancer, melanoma, and lung cancer.¹⁶⁻¹⁸ Soung et al first reported a mutation of the *ErbB4* kinase domain in invasive ductal breast carcinoma.¹⁶ Prickett et al performed a mutational analysis of the 86 protein tyrosine kinase genes, and found *ErbB4* mutations in 19% of individuals with metastatic melanoma. In addition, they demonstrated that the mutations enhanced the kinase activity and transformation activity of ErbB4 in melanoma cells.¹⁷ Kurppa et al investigated *ErbB4* mutations that were previously reported in non-small-cell lung cancer, and revealed that some mutations increased the kinase activity and facilitated dimerization of ErbB4.¹⁸ These studies demonstrated the complicated functions of ErbB4, and also suggested the possibility of *ErbB4* mutations as effective targets in malignant tumors.

Tumor cells consume a large amount of nutrients and oxygen to sustain cell proliferation, and angiogenesis, a system that supplies nutrients and oxygen to tumor tissues via neovascularization, has been recognized to be a well established target. However, as the effects of anti-angiogenesis drugs were insufficient and some patients acquired resistance to the treatment,¹⁹ alternative blood supply pathways have been implied. VM, a vascular-like network structure that is formed only by cancer cells, has received much attention over the past 2 decades.²⁰ VM is considered to be a novel blood supply system that facilitates tumor growth and metastasis, and is observed in many aggressive tumors.²¹ A previously conducted meta-analysis study revealed that VM is significantly associated with a poor prognosis in cancer patients,²² suggesting that the inhibition of VM could provide an ideal strategy for cancer treatments. Essential factors of VM have been identified in recent years, and there is mounting evidence that cell surface receptors, such as VEGFR, VE-cadherin, EphA2, and integrin β 1, regulate VM formation.²³⁻²⁶ By interacting with high-affinity ligands, such as growth factors or extracellular matrix substrates, these receptors activate the intracellular signals that contribute to VM formation. Although ErbB4 mediates various types of intracellular signaling pathways by ligand stimulation, and mutations of the *ErbB4* gene are associated with metastatic phenotypes, the effect of ErbB4 on VM formation has not yet been studied.

In this study, we investigated the role of ErbB4 on VM formation in human breast cancer cell lines. Using pharmacological and genetic approaches, we demonstrated that the expression of ErbB4 is negatively correlated with VM formation, and that activated ErbB4 improves the VM formation capability of breast cancer cells.

2 | MATERIALS AND METHODS

2.1 | Reagents

Neuregulin 1- β 1 was purchased from R&D Systems (#396-HB-050; Minneapolis, MN) and dissolved in PBS containing 0.5% bovine serum albumin. Afatinib (BIBW2992) was purchased from Tokyo Chemical Industry (#A2870; Tokyo, Japan) and dissolved in dimethyl sulfoxide.

2.2 | Cell cultures

Human breast cancer cell lines MCF-7, MDA-MB-231, and T47D were obtained from the RIKEN BioResource Research Center (Tsukuba, Japan). Cells were maintained in Dulbecco's modified Eagle medium (Nissui Pharmaceutical #05919; Tokyo, Japan) supplemented with 7% (v/v) fetal bovine serum, 100 U/mL penicillin G, 100 mg/L kanamycin, 600 mg/L L-glutamine, and 2.25 g/L NaHCO₃ at 37°C in an humidified incubator with 5% CO₂. All cell lines were tested for the presence of *Mycoplasma* contamination using Hoechst 33258 staining (Polysciences #09460-100; Warrington, PA).

2.3 | Plasmid construction

Full-length cDNA encoding wild-type human ErbB4 (NM_005235) was amplified from the pcDNA3.1-ErbB4 vector (gifted by Yardena Samuels, Addgene #29527; Watertown, MA) by polymerase chain reaction (PCR) with the following primers: forward, 5'-TTTTACGC GTATGAAGCCGCGACAGGAC-3', and reverse, 5'-TTTTGCGGCC GCTTACACCACAGTATTCCGGTG-3'. Considering its potential usefulness in future experiments, we generated Cas9-resistant ErbB4 by inverse PCR with the following primers: forward, 5'-GTCTCTT TGCTGGTCCGCCCGGGACCGTCCAGCCCAGCGATTCTC-3', and reverse, 5'-CCACACCCACAAGCCGGTGGCTGGCTTCATACGCGTG AATTCTCG-3'. Point mutations in the ErbB4 cDNA were generated using a site-directed mutagenesis method. The E872K mutation was introduced by overlap extension PCR with the following primers: forward, 5'-GAAGGAGATAAAAAGAGTACAATGCTGATGGAGG-3', and reverse, 5'-ACTCTTTTATCTCCTTCCAAGAGTCTGGCTAG C-3'. The K751M mutation was introduced by inverse PCR with the following primers: forward, 5'-TGATTCTTAATGAGACAAGTGGTCC CAAGG-3', and reverse, 5'-TAATAGCCACAGGAATCTTACAGTTT CTC-3'. The resultant DNAs were subcloned into the *Mlu*I/*Not*I restriction sites of the pCI-neo vector (Promega #E1841; Madison, WI).

2.4 | Establishment of ErbB4-overexpressing cell lines

MDA-MB-231 cells were transfected with ErbB4 expression vectors using Lipofectamine™ 3000 Transfection Reagent (Thermo Fisher Scientific #L3000008; Waltham, MA) according to the manufacturer's instructions. Transfected cells were treated with 500 µg/mL G418 (FUJIFILM Wako Pure Chemical #074-05963; Osaka, Japan) to select the stably ErbB4-overexpressing cells, then clonal cell lines were obtained by limiting dilution.

2.5 | CRISPR/Cas9-mediated ErbB4 knockout in the T47D cell line

Gene knockout (KO) was accomplished using the clustered regularly interspaced palindromic repeat (CRISPR)/CRISPR-associated protein 9 (Cas9) system as previously described.^{26,27} To minimize off-target effects, we used the pSpCas9n(BB)-2A-Puro (PX462) V2.0 plasmid (gifted by Feng Zhang, Addgene #62987) for the co-expression of Cas9n (D10A nickase mutant). Targets of 2 guide RNAs were designed within exon 1 of *ErbB4*, and the sequences of the oligonucleotides used were as follows: forward 1, 5'-CACCGCAGACCCAAAGT CCTGTCCG-3', and reverse 1, 5'-AAACGCGACAGACTTTGGGTCTG C-3'; and forward 2, 5'-CACCGGTGAGCCTTCTCGTGGCGGC-3', and reverse 2, 5'-AAACGCCGCCACGAGAAGGCTACC-3'. Each pair of oligonucleotides was annealed and subsequently inserted into the *Bbs*I restriction site of the Cas9n expression vector.

T47D cells were co-transfected with ErbB4-CRISPR/Cas9 vectors using Lipofectamine™ 3000 Transfection Reagent according to the manufacturer's instructions. Transfected cells were treated with 2 µg/mL puromycin dihydrochloride (Merck KGaA #P8833; Darmstadt, Germany), then clonal cell lines were established by limiting dilution.

2.6 | Western blot

Western blot was performed as previously described.^{26,28-31} Cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% [w/v] sodium dodecyl sulfate (SDS), 1% [v/v] Triton X-100, 1% [w/v] sodium deoxycholate, and 1 mM phenylmethylsulfonyl fluoride) with the phosphatase inhibitor cocktail PhosSTOP (Merck KGaA #4906845001) on ice. Lysed cells were sonicated for 15 sec, and the protein concentrations were measured by Coomassie Brilliant Blue G-250 staining (Bio-Rad Laboratories #5000006JA; Hercules, CA). Loading buffer (350 mM Tris-HCl, pH 6.8, 30% [w/v] glycerol, 0.012% [w/v] bromophenol blue, 6% [w/v] SDS, and 30% [v/v] 2-mercaptoethanol) was added to each lysate, which was subsequently boiled for 5 min and subjected to SDS-polyacrylamide gel electrophoresis. Proteins were transferred to a polyvinylidene difluoride membrane and immunoblotted with anti-EGFR (Cell Signaling Technology #2232; Danvers, MA), anti-HER2 (Santa Cruz Biotechnology #sc-7301; Dallas, TX), anti-ErbB3 (Cell Signaling

Technology #12708), anti-ErbB4 (Cell Signaling Technology #4795), anti-p-ErbB4 (Y1284; Cell Signaling Technology #4757), or anti- α -tubulin (Merck KGaA #T5168) antibody. The secondary antibodies used were HRP-linked-anti-mouse IgG (Cytiva #NA931; Marlborough, MA) and HRP-linked-anti-rabbit IgG (Cytiva #NA934). Signals were detected with the enhanced chemiluminescence reagent Immobilon (Merck KGaA #WBKLS0500) or Lightning Plus-ECL (PerkinElmer #NEL104001EA; Waltham, MA).

2.7 | In vitro VM formation assay

The in vitro VM formation assay was performed as previously described.^{25,26,32,33} A 96-well plate was coated with 40 µL/well of Matrigel® Growth Factor Reduced (Corning #354230; Corning, NY) and allowed to gelate for 30 min at 37°C. The cells suspended in serum-free Dulbecco's-modified Eagle's medium were seeded onto the Matrigel at 1.6×10^4 cells/well, and cultured at 37°C in an humidified incubator containing 5% CO₂. We defined capillary-like tubes by VM as areas surrounded by cells. The formed tubes were photographed using phase-contrast microscopy (Leica #DMI1 MC120, Wetzlar, Germany), and the number of tubes was counted in 6 randomly selected independent fields.

2.8 | Statistical analysis

Differences between the 2 groups were analyzed using two-tailed Student *t* test. Three- or more groups datasets were analyzed by one-way ANOVA with Tukey's test using SPSS statistics software (version 27; IBM, Armonk, NY). The results were expressed as the means \pm standard deviation (SD). *P*-values of $< .05$ were considered to be statistically significant.

3 | RESULTS

3.1 | Expression of ErbB4 delays VM formation

To evaluate the role of ErbB4 on VM formation, we firstly sought to characterize the expression of the ErbB receptors in human breast cancer MDA-MB-231 and T47D cells. Western blot analysis revealed that MDA-MB-231 (ErbB4-negative) and T47D (ErbB4-positive) cells highly expressed EGFR and ErbB3, respectively (Figure 1A). Previous studies have demonstrated that VM-positive tumor cells exhibited network formation under Matrigel-containing culture conditions,^{20,33} so we examined the potential of network formation using an in vitro VM formation assay. Our results showed that MDA-MB-231 cells, but not T47D cells, formed VM networks (Figure S1A,B). In addition, ErbB4-highly expressing MCF-7 cells did not form the network structures (Figure S1A,B), implying that high expression of ErbB4 was associated with the VM-negative phenotype in human breast cancer cells.

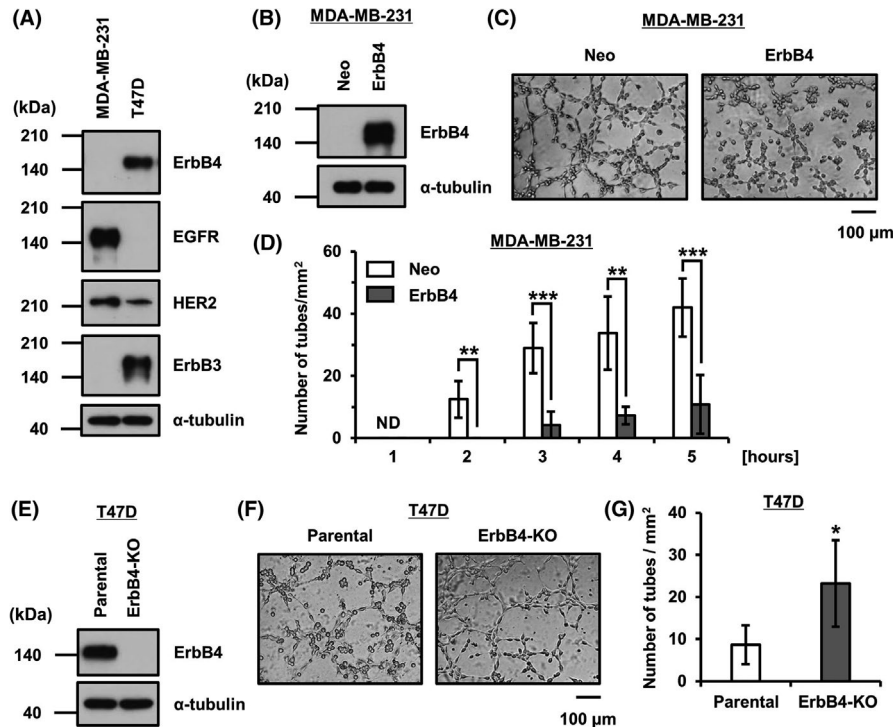


FIGURE 1 Expression of ErbB4 delays vasculogenic mimicry (VM) formation in human breast cancer cells. A, Human breast cancer MDA-MB-231 and T47D cells were lysed and immunoblotted with the indicated antibodies. B, MDA-MB-231 cells stably overexpressing ErbB4 were established, and the expression of the protein was confirmed by western blot. The pCI-neo empty vector (Neo) was used as a control. C and F, Cells were suspended in serum-free medium, and seeded onto Matrigel-precoated wells. Representative images of VM formation in MDA-MB-231 (C) and T47D (F) cells are shown (3 h after seeding). D and G, VM formation was quantified every hour (D) or 3 h (G) after seeding, and the number of tubes was counted in 6 randomly selected independent fields. E, T47D cells were co-transfected with ErbB4-CRISPR/Cas9 vectors, and ErbB4-knockout (ErbB4-KO) cell lines were established. Depletion of the ErbB4 protein was confirmed by western blot. Scale bars, 100 μ m. Data shown are the means \pm SD. *P*-values < .05, < .01, and < .001 are indicated by *, **, and ***, respectively. ND, not detected

To confirm the effect of ErbB4 expression, we established stably ErbB4-overexpressing MDA-MB-231 cells (Figure 1B). The *in vitro* VM assay demonstrated that the number of tubes formed by cells gradually increased in the control cells; however, the ErbB4-overexpressing cells significantly delayed network formation (Figure 1C,D). Interestingly, because the expression of ErbB4 did not completely abrogate the VM formation, the early stage of VM formation appeared to be negatively regulated by ErbB4.

As ectopic expression of ErbB4 suppressed VM formation, we assessed whether endogenous ErbB4 also acted as a VM suppressor. We performed CRISPR/Cas9-mediated *ErbB4* gene deletion in T47D cells, and clonal ErbB4-KO cell lines were established (Figure 1E). Consistent with the results of MDA-MB-231 cells, ErbB4 depletion enhanced the VM formation capability of T47D cells (Figure 1F,G). We also examined the effect of *ErbB4* gene disruption on VM formation in MCF-7 cells. We confirmed that KO of endogenous ErbB4 changed the MCF-7 cells to have VM-like phenotypes; however, the parental MCF-7 cells did not exhibit any obvious stable tube formation (Figure S1C,D). These data suggested that the expression of ErbB4 delayed the VM formation in human breast cancer cells.

3.2 | NRG1 promotes ErbB4-mediated VM formation

Neuregulin 1 (NRG1), a ligand of ErbB4, promotes the phosphorylation of ErbB4 and migration in T47D cells.³⁴ Therefore, we hypothesized that NRG1-mediated ErbB4 signaling had positive effects on VM formation. To evaluate whether the expression or activity of ErbB4 was more important for VM formation, we overexpressed wild-type (wt), constitutively active E872K, and kinase-dead K751M mutant ErbB4 in MDA-MB-231 cells (Figures 2A and S2). Treatment with 100 ng/mL NRG1 phosphorylated/activated ErbB4 (wt) and accelerated VM formation (Figure 2B-D). In contrast, treatment with NRG1 resulted in neither the phosphorylation of ErbB4 (K751M) nor an increase in the number of tubes (Figure 2B-D), suggesting that the NRG1-mediated activation of ErbB4 facilitated VM formation.

We also treated T47D cells with NRG1 to stimulate endogenous ErbB4. Compared with the MDA-MB-231 cells, T47D cells were sensitive to NRG1 at around 30 ng/mL, and ErbB4 phosphorylation was significantly promoted (Figure 2E). Treatment with NRG1 resulted in a two-fold increase in the number of VM tubes in the T47D cells (Figure 2F,G). Furthermore, we confirmed that NRG1 did not accelerate VM formation in both parental MDA-MB-231 cells

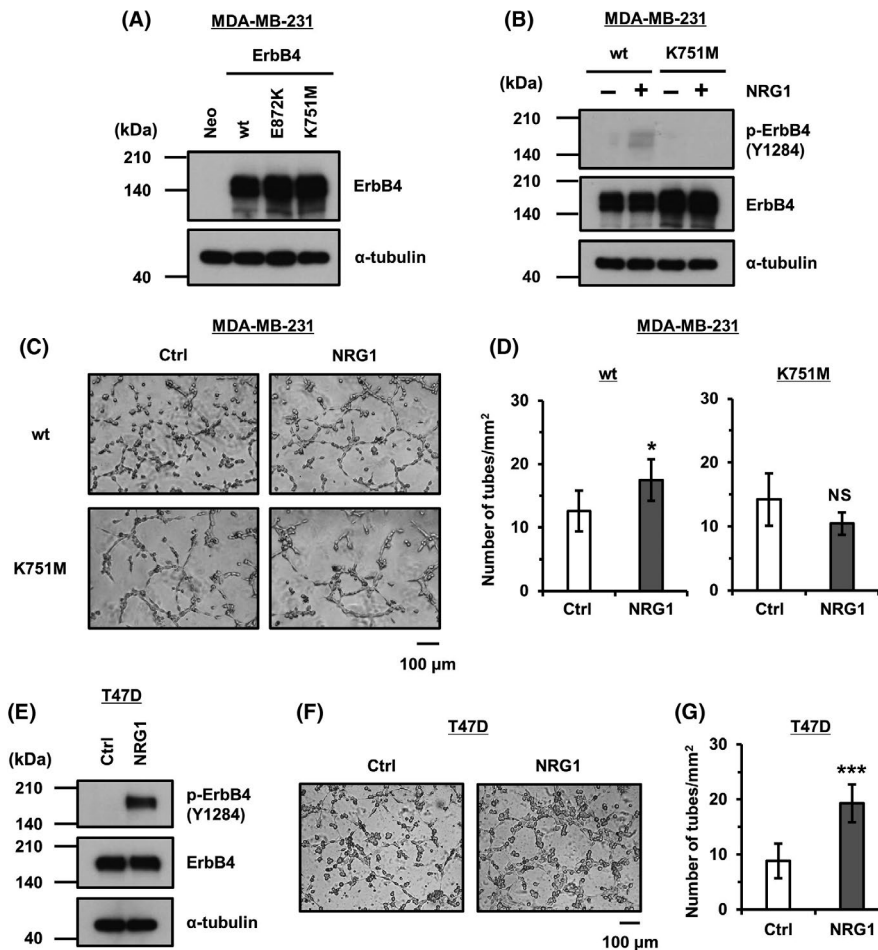


FIGURE 2 NRG1 promotes ErbB4-mediated vasculogenic mimicry (VM) formation. A, MDA-MB-231 cells stably expressing wild-type (wt), E872K, or K751M ErbB4 were established, and the expression of the proteins was confirmed by western blot. B and E, ErbB4-overexpressing MDA-MB-231 (B) and parental T47D (E) cells were stimulated with vehicle control or NRG1 (B, 100 ng/mL; E, 30 ng/mL) for 30 min in serum-free medium. The stimulated cells were lysed and immunoblotted with the indicated antibodies. C and F, Cells were suspended in serum-free medium and treated with vehicle control or NRG1 (C, 100 ng/mL; F, 30 ng/mL), then seeded onto Matrigel-precoated wells. Representative images of VM formation in ErbB4-overexpressing MDA-MB-231 (C) and parental T47D (F) cells are shown. D and G, VM formation in ErbB4-overexpressing MDA-MB-231 (D) and parental T47D (G) cells was quantified at 5 h after seeding, and the number of tubes was counted in 6 randomly selected independent fields. Scale bars, 100 μ m. Data shown are the means \pm SD. *P*-values < .05 and < .001 are indicated by * and ***, respectively. NS, not significant

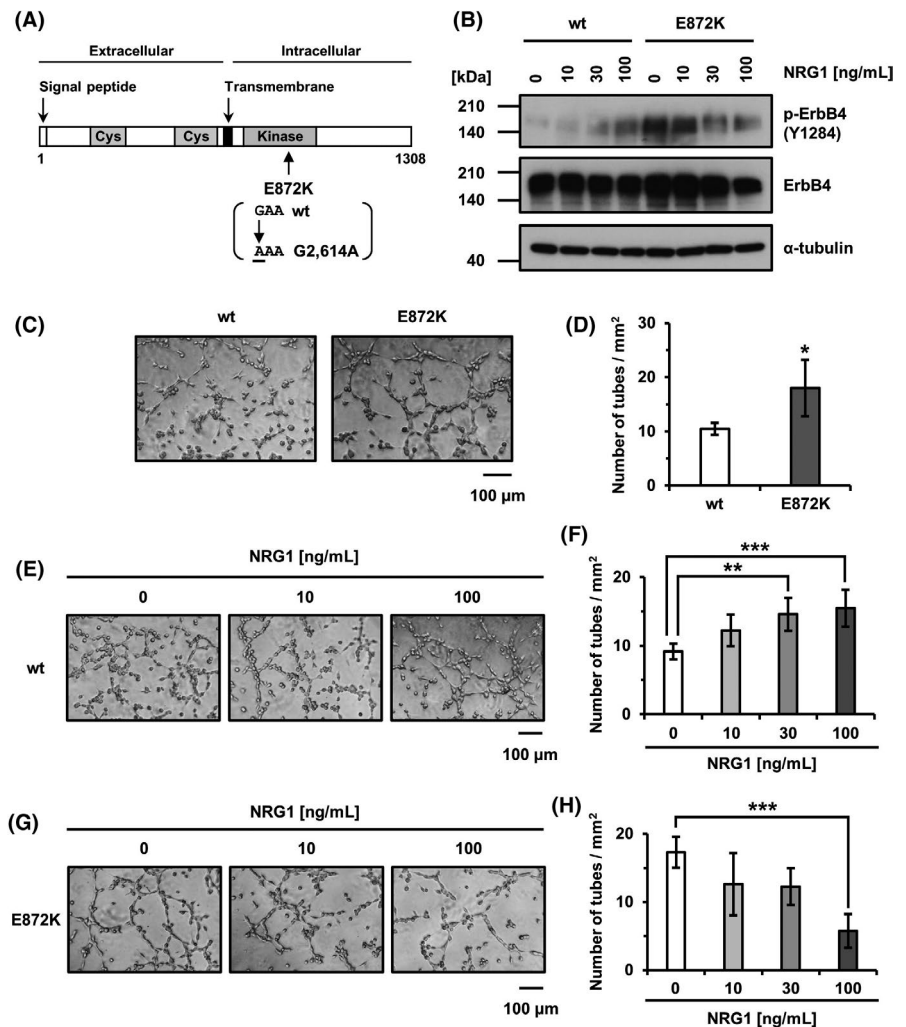
and ErbB4-KO T47D cells (Figure S3). Collectively, these results indicated that ErbB4 enhanced VM formation via ligand-mediated phosphorylation/activation in breast cancer cells.

3.3 | E872K mutation enhances ErbB4-mediated VM formation in the absence of NRG1

E872K is one of the *ErbB4* mutations caused by the substitution of G2614A, and the mutation was identified in 2 types of cancer: cutaneous metastatic melanoma and invasive ductal breast carcinoma (Figure 3A).^{16,17} Therefore, we evaluated the potential role of the E872K mutation, and established E872K mutation-expressing MDA-MB-231 cells (Figure 2A). We found that the E872K mutation enhanced the basal phosphorylation levels in the absence of

NRG1; however, phosphorylation of ErbB4 (E872K) was attenuated by treatment with NRG1 (Figure 3B). A previous study demonstrated that multiple C-terminal tyrosine residues of EGFR are constitutively phosphorylated in MDA-MB-468 cells, and these phosphorylation are downregulated by Erk-mediated negative feedback in EGF-treated or heregulin-treated cells.³⁵ Because the Y1284 residue of E872K ErbB4 is phosphorylated constitutively, our results indicated that a similar negative feedback was induced by NRG1 treatment in E872K ErbB4-overexpressing cells. Consistent with the phosphorylation states, the E872K mutation facilitated ErbB4-mediated VM formation in the absence of NRG1 (Figure 3C,D). Moreover, we confirmed a dose-dependent increase in the number of tubes in ErbB4 (wt)-overexpressing cells (Figure 3E,F), whereas the E872K mutation decreased the tube numbers in the presence of NRG1 (Figure 3G,H). Similarly,

FIGURE 3 E872K mutation enhances ErbB4-mediated vasculogenic mimicry (VM) formation in the absence of NRG1. **A**, Schematic diagram of the functional domain of human ErbB4. Two cysteine-rich and tyrosine kinase domains are contained in the extracellular and intracellular regions, respectively. The partial nucleotide sequences of the wild-type (wt) and E872K *ErbB4* are shown. The substituted nucleotide is underlined. **B**, The wt or E872K ErbB4-overexpressing MDA-MB-231 cells were stimulated with vehicle control or NRG1 (10, 30, or 100 ng/mL) for 30 min in serum-free medium. The stimulated cells were lysed and immunoblotted with the indicated antibodies. **C**, **E**, and **G**, Cells were suspended in serum-free medium and treated with or without several concentrations of NRG1 (**E** and **G**), then seeded onto Matrigel-precoated wells. Representative images of VM formation are shown. **D**, **F**, and **H**, VM formation was quantified at 5 h after seeding, and the number of tubes was counted in 6 randomly selected independent fields. Scale bars, 100 μ m. Data shown are the means \pm SD. *P*-values < .05, < .01 and < .001 are indicated by *, **, and ***, respectively



VM formation was not promoted by a high concentration of NRG1 (300 ng/mL) in T47D cells (Figure S4A,B), suggesting that excessive NRG1 stimulation had negative effects on VM formation. These results indicated that activated ErbB4 promoted VM formation, and suggested that ErbB4 harboring activate mutations may be a promoter of VM in the absence of NRG1, whereas an excessive ErbB4-mediated signal might be a negative factor for VM formation.

3.4 | Afatinib treatment on ErbB4-mediated VM formation

Inhibitors targeting ErbB receptors have been developed and used for cancer treatment; however, little information is known about their effects on VM-positive cancer cells. To investigate whether ErbB inhibitors can abrogate VM formation, we used the pan-ErbB inhibitor afatinib. Although low-dose afatinib did not affect the VM formation in parental MDA-MB-231 cells, 3 μ M afatinib significantly inhibited the network structures (Figure 4A,B). In addition, we examined ErbB4-overexpressing MDA-MB-231 cells, and revealed that NRG1/ErbB4 (wt)-mediated VM formation was also suppressed

by 3 μ M afatinib that could inhibit NRG1-stimulated ErbB4 (wt) phosphorylation (Figures 4C,D and S5A), suggesting that ErbB inhibitors can inhibit VM formation regardless of the expression of ErbB4. Several mutations of ErbB4 induce increased sensitivity to lapatinib in melanoma¹⁴; therefore, we evaluated whether the VM formation of E872K ErbB4-overexpressing cells would be abrogated by a low-dose administration of afatinib. We found that the cells with the E872K mutation exhibited increased sensitivity to afatinib, because treatment with 0.1 μ M afatinib significantly suppressed both E872K ErbB4 phosphorylation and VM formation (Figures 4E,F and S5B); this indicated that the ErbB4-expressing cells harboring the E872K mutation acquired 2 properties: VM formation capability and vulnerability to ErbB inhibitors.

4 | DISCUSSION

As the aberrant activation of RTKs is closely related to malignancies in many tumors, the elucidation of their biological functions is important. The ErbB receptors are RTKs that are highly expressed in various cancer cells, and the inhibition of ErbB receptors has therefore been considered an effective strategy for cancer patients.

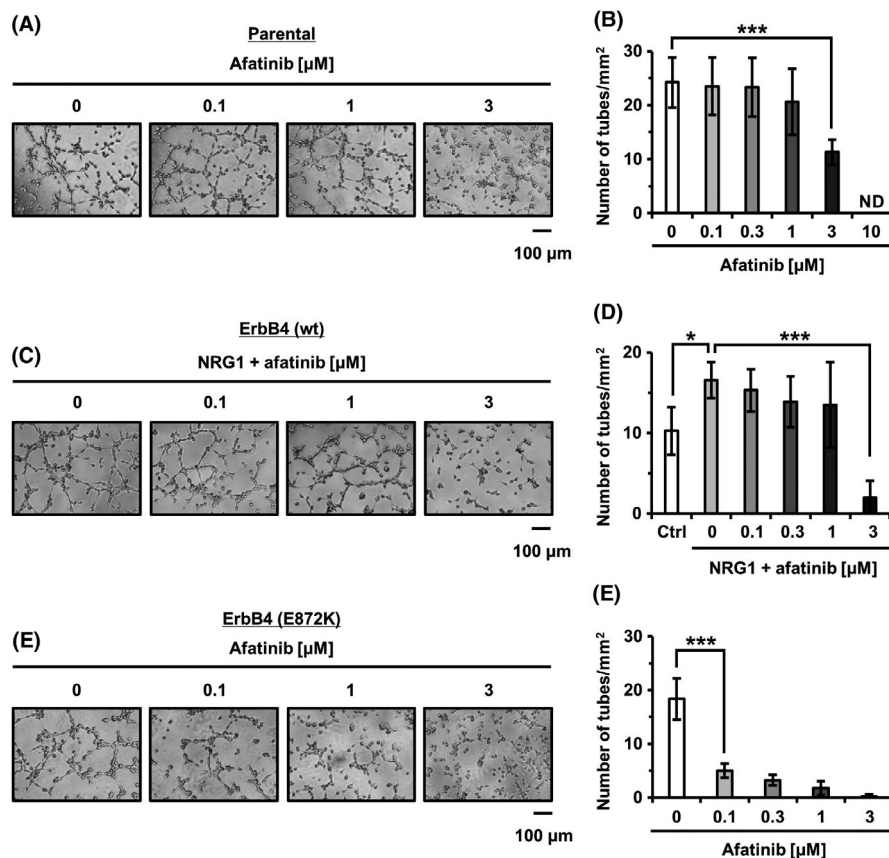


FIGURE 4 Afatinib inhibits ErbB4-mediated vasculogenic mimicry (VM) formation. A, Parental MDA-MB-231 cells were suspended in serum-free medium and treated with dimethyl sulfoxide or afatinib (0.1, 0.3, 1, 3, or 10 μM), then seeded onto Matrigel-precoated wells. Representative images of VM formation in parental MDA-MB-231 cells are shown (3 h after seeding). B, D, and F, VM formation in parental (B) and ErbB4-overexpressing (D, F) MDA-MB-231 cells was quantified at 5 h after seeding, and the number of tubes was counted in 6 randomly selected independent fields. C, The wild-type (wt) ErbB4-overexpressing MDA-MB-231 cells were suspended in serum-free medium and co-treated with afatinib and 100 ng/mL NRG1, then seeded onto Matrigel-precoated wells. Representative images of VM formation are shown. E, E872K ErbB4-overexpressing MDA-MB-231 cells were suspended in serum-free medium and treated with afatinib, then seeded onto Matrigel-precoated wells. Representative images of VM formation are shown. Scale bars, 100 μm . Data shown are the means \pm SD. *P*-values < .05, < .01, and < .001 are indicated by *, **, and ***, respectively. ND, not detected

Although EGFR and HER2 have been studied extensively, and specific inhibitors targeting these proteins have been approved as anti-cancer drugs,⁴ the oncogenic functions of ErbB4 have not yet been fully defined. In the present study, we focused on VM, which contributes to tumor malignancy, and aimed to elucidate the effects of ErbB4 on VM formation in human breast cancer cell lines.

Although ErbB4 is highly expressed in T47D cells, we could not detect the endogenous expression of ErbB4 in MDA-MB-231 cells (Figure 1A). Given that MDA-MB-231 cells, but not T47D cells, have a potent network formation capability (Figure S1B), the expression of ErbB4 seems to play a negative role for VM formation in breast cancers. Therefore, we generated ErbB4-overexpressing cells, and confirmed the abrogation of VM formation in these cells (Figure 1B-D). It has been reported that transmembrane receptors activate intracellular signaling and induce the expression of several genes that lead to the transformation to VM-positive phenotypes.^{33,36-38} Due to the localization of ErbB4 proteins on the cell membrane, it is possible that the functions of the receptors that promote VM formation

were physically disturbed, and the subsequent signal activation was delayed. Therefore, the presence of ErbB4 could not completely abolish VM formation.

We also examined endogenous ErbB4 by CRISPR/Cas9-mediated gene deletion, and demonstrated that depletion of ErbB4 improved VM formation capability (Figures 1B-D and Figure S1C,D). Endogenous ErbB4 is complicated, because there are 4 variants that are characterized by the extracellular juxtamembrane (JM) and intracellular cytoplasmic (CYT) domains.³⁹ The JM-a isoform has a proteinase cleavage site, and an 80-kDa ICD is released upon ligand binding.^{12,40} Because we transfected MDA-MB-231 cells with the JM-a CYT-1 isoform of ErbB4, we detected the ICD fragment in the absence of NRG1 (data not shown). In contrast, we could not detect the ICD even with NRG1-mediated stimulation in T47D cells (data not shown), suggesting that endogenous ErbB4 may be the JM-b isoform in T47D cells. Therefore, both the JM-a and JM-b isoforms appear to play negative roles in VM formation in human breast cancer cells.

NRG1-stimulated ErbB4 activation mediates various signaling pathways and cellular plasticity as well as other ErbB receptors^{14,36}; therefore, we activated ErbB4 with NRG1, and examined whether phosphorylation/activation would alter the VM formation capability. Treatment with NRG1 induced the phosphorylation of ErbB4 and activated VM formation (Figure 2B-D). However, the kinase-dead K751M mutant was not stimulated by NRG1, suggesting that the kinase activity of ErbB4 promoted VM formation. In addition, NRG1 also activated VM formation in T47D cells (Figure 2E-G). MDA-MB-231 and T47D cells are distinct types of breast cancer cell lines; as shown in Figure 1A, in addition to the difference in ErbB4 expression, they showed differences in EGFR and ErbB3 expression, ie, EGFR is more highly expressed in MDA-MB-231 cells, whereas ErbB3 is more highly expressed in T47D cells. ErbB receptors form homodimers or heterodimers, and induce transphosphorylation by ligand binding.⁴¹ NRG1 binds to ErbB3 as well as to ErbB4⁴²; therefore, T47D cells tend to be more activated by NRG1 treatment when compared with MDA-MB-231 cells. Consistent with this, NRG1 significantly induced ErbB4 phosphorylation and improved VM formation in T47D cells. However, it is also possible that ErbB4 interacts with EGFR in MDA-MB-231 cells. It has been reported that an aptamer that targets the EGFR-integrin $\alpha_v\beta_3$ complex impairs VM formation in triple-negative breast cancer⁴³; therefore, EGFR is considered to be involved in VM formation by MDA-MB-231 cells.

ErbB4 is frequently mutated in cutaneous melanoma and non-small-cell lung cancer^{17,18}; it has been reported that almost all of these *ErbB4* mutations increased the phosphorylation level of ErbB4 in the absence or presence of NRG1, and conferred oncogenic properties to the cells. However, Vidal et al⁴⁴ reported that a constitutively active I658E mutation enhanced ErbB4-mediated cell-killing activity. As phosphorylated ErbB4 promotes both cell proliferation and apoptosis, the phosphorylation state is not enough to determine the role of *ErbB4* mutations in tumor cells. In this study, we focused on the E872K mutation of *ErbB4*, because the mutation is located in the protein tyrosine kinase domain, and is found in melanoma and breast cancer patients.^{16,17} We overexpressed E872K ErbB4 in MDA-MB-231 cells, and found that the basal phosphorylation level increased (Figure 3B). Interestingly, NRG1 stimulation caused a decrease of ErbB4 phosphorylation. Given that constitutively phosphorylated tyrosine is downregulated in EGFR-overexpressing cells stimulated with EGF,³⁵ the E872K mutation of *ErbB4* is considered to cause a similar negative feedback. Although Tvorogov et al showed that the E872K mutation did not enhance the phosphorylation of ErbB4 in MCF-7 cells,⁴⁵ because endogenous ErbB3 and ErbB4 are highly expressed in the MCF-7 cells, their result may have been influenced by negative feedback. Many gene alterations have been detected in RTKs,^{46,47} and constitutively activated RTKs are a critical target for cancer chemotherapy. In this study, we revealed that the E872K mutation of ErbB4 enhanced VM formation in the absence of NRG1 in MDA-MB-231 cells (Figure 3C,D). Whereas a previous report indicated that gain-of-function mutations of ErbB4 promoted cell proliferation in melanoma, the mutations also increased the

vulnerability of the cells to ErbBs inhibitors.¹⁷ Our results showed that E872K ErbB4-mediated VM formation was inhibited by low-dose afatinib (Figure 4C). Consistent with the previous mutational study, *ErbB4* mutations appeared to confer both oncogenic activity and increased sensitivity to tyrosine kinase inhibitors.

Many studies have demonstrated that ErbB receptors regulate cell growth, survival, and motility, therefore we evaluated the effects of ErbB4 on these cell functions in MDA-MB-231 cells (Figure S2). As the results, overexpression of wt ErbB4 promoted cell proliferation and E872K mutation enhanced this activity (Figure S2A). Because K751M ErbB4 did not stimulate cell growth, it was indicated that kinase activity is critical for ErbB4-mediated cell proliferation. As shown in Figure S2B,C, the wound-healing assay revealed that ErbB4 downregulated cell migration in MDA-MB-231 cells. In addition, E872K ErbB4 showed high migration ability compared with wt ErbB4. These results corresponded to the VM formation capability, therefore it was suggested that ErbB4 suppresses VM formation by downregulating cell motility. Furthermore, we examined anchorage-independent cell survival evaluated by using poly-HEMA-coated well plates, and found that ErbB4 significantly enhanced its activity in MDA-MB-231 cells (Figure S2D). Collectively, these results demonstrated that ErbB4 enhances cell growth, and supported the idea that kinase activity might be crucial for ErbB4-mediated VM formation.

Breast cancer is classified into some groups according to the expression levels of the estrogen receptor (ER), progesterone receptor (PR), and HER2. In this study, we used MDA-MB-231 (triple-negative: ER-PR-HER2-), MCF-7 (Luminal A: ER+PR+HER2-), and T47D (Luminal A: ER+PR+HER2-) cells. ER and PR are critical to the outcome of breast cancer patients, therefore we examined whether these hormone receptors affected VM formation. As MCF-7 is present in VM-negative tumor cells, we speculated that ER and/or PR signaling might negatively regulate VM formation. As a result, treatment with fulvestrant (ER antagonist) and mifepristone (PR antagonist) did not induce VM formation in MCF-7 cells (data not shown). Therefore, we concluded that ER and PR do not regulate VM formation in MCF-7 cells. Moreover, we investigated the effect of fulvestrant and mifepristone on VM formation in T47D cells. Treatment with fulvestrant decreased the number of tubes, whereas mifepristone did not affect VM formation in T47D cells (data not shown). Taken together, these results indicated that the contribution of the ER and PR signaling pathways for VM formation is cell dependent.

Our findings indicated that expression of ErbB4 was associated with the VM-negative phenotype in human breast cancer cells. However, NRG1-mediated phosphorylation of ErbB4 converted cells from the VM-negative phenotype to the VM-positive phenotype, and the E872K mutation enhanced VM formation in the absence of NRG1. The ErbB inhibitor afatinib suppressed VM formation, and the effects of the E872K mutation were effectively inhibited by low-dose afatinib treatment. Taken together, ErbB4 appears to regulate VM formation, and mutations of the *ErbB4* gene are a promising target for VM-harboring cancers.

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DISCLOSURE

The authors have no conflicts of interest to report.

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