Long isoform of VEGF stimulates cell migration of breast cancer by filopodia formation via NRP1/ARHGAP17/Cdc42 regulatory network

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VEGF stimulates endothelial cells as a key molecule in angiogenesis. VEGF also works as a multifunction molecule, which targets a variety of cell members in the tumor microenvironment. We aimed to reveal VEGF-related molecular mechanisms on breast cancer cells. VEGF-knocked-out MDA-MB-231 cells (231VEGFKOex) showed rounded morphology and shorter perimeter (1.6-fold, p < 0.0001). The 231VEGFKOex cells also showed impaired cell migration (2.6-fold, p = 0.002). Bevacizumab treatment did not induce any change in morphology and mobility. Soluble neuropilin-1 overexpressing MDA-MB-231 cells (231SNRP1) exhibited rounded morphology and shorter perimeter (1.3-fold, p < 0.0001). The 231SNRP1 cells also showed impaired cell migration (1.7-fold, p = 0.003). These changes were similar to that of 231VEGFKOex cells. As MDA-MB-231 cells express almost no VEGFR, these results indicate that the interaction between NRP1 and long isoform of VEGF containing a NRP-binding domain regulates the morphology and migration ability of MDA-MB-231 cells. Genome-wide gene expression profiling identified ARHGAP17 as one of the target genes in the downstream of the VEGF/NRP1 signal. We also show that VEGF/NRP1 signal controls filopodia formation of the cells by modulating Cdc42 activity via ARHGAP17. Among 1,980 breast cancer cases from a public database, the ratio of VEGF and SEMA3A in primary tumors (n = 450) of hormone-receptor-negative breast cancer is associated with ARHGAP17 expression inversely, and with disease free survival. Altogether, the bevacizumab-independent VEGF/NRP1/ARHGAP17/Cdc42 regulatory network plays important roles in malignant behavior of breast cancer.

Key words: breast cancer, VEGF, neuropilin, ARHGAP17, filopodia, migration, cdc42, isoform

Abbreviations: BC: breast cancer; DFS: disease free survival; ECOG: Eastern Cooperative Oncology Group; ER: estrogen receptor; HR: hormone receptor; NRP1: neuropilin1; OS: overall survival; PFS: progression-free survival; SEMA3A: Semaphorin3A; sNRP1: soluble neuropilin1; TNBCs: triple negative breast cancers

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Introduction

Breast cancer (BC) is one of the most common forms of cancer, with more than 1,300,000 new cases and 450,000 deaths each year worldwide.¹ BC is classified into several subtypes, such as the estrogen receptor (ER) positive (luminal) subtype, HER2-enriched subtypes and triple-negative (TN) subtypes. TN breast cancers (TNBCs) are highly metastatic tumors with poor prognosis.² Approximately 15% of BC patients develop distant metastasis, which is responsible for about 90% of BC-associated mortality.³ Therefore, it is important to understand the mechanisms underlying BC metastasis. Metastasis is a complex process that includes tumor cell invasion/migration/motility, intravasation, survival in blood or lymphatic circulation (resistant to anoikis), extravasation and regrowth in new environments.⁴ However, the critical molecular mechanisms underlying tumor metastasis remain unclear.

Angiogenesis plays important roles in tumor progression. VEGF (also known as VEGF-A), secreted by tumor and stromal cells, enhances angiogenesis in tumor microenvironment.⁵ Newly formed vessels provide oxygen and nutrients to assist tumor growth. Clinically, VEGF expression in BC tumors has been reported to be associated with worse prognosis.⁶ Bevacizumab, an antibody that neutralizes VEGF, suppresses tumor
Vascular endothelial growth factor (VEGF) acts on endothelial cells to promote blood vessel formation and tumor growth. In breast cancer, VEGF expression is associated with poor prognosis, though its effects on breast cancer cells are not well understood. Here, the long isoform of VEGF (VEGF165) was found to generate a signal through the transmembrane protein neuropilin-1 (NRP1), without acting on VEGF receptors. ARHGAP17, a RhoGAP family protein that regulates Cdc42 activity and controls filopodia formation and cell migration ability, was identified as a VEGF165 signaling target. The VEGF/NRP1/ARHGAP17 network likely contributes to malignant cell behavior in hormone receptor-negative breast cancer.

What’s new?
Vascular endothelial growth factor (VEGF) acts on endothelial cells to promote blood vessel formation and tumor growth. In breast cancer, VEGF expression is associated with poor prognosis, though its effects on breast cancer cells are not well understood. Here, the long isoform of VEGF (VEGF165) was found to generate a signal through the transmembrane protein neuropilin-1 (NRP1), without acting on VEGF receptors. ARHGAP17, a RhoGAP family protein that regulates Cdc42 activity and controls filopodia formation and cell migration ability, was identified as a VEGF165 signaling target. The VEGF/NRP1/ARHGAP17 network likely contributes to malignant cell behavior in hormone receptor-negative breast cancer.

In general, BC cells express almost no or very low level of VEGFR1/2. However, BC cells/tissues as well as other malignancies express various amounts of other VEGF receptors, neuropilins-1/2. One neuropilin, NRP1, is a transmembrane protein that consists of a large extracellular domain, one transmembrane domain, and a relatively short cytoplasmic tail. The extracellular domains of NRP1 are divided into the a1–a2 subdomain, which binds semaphorins, the b1–b2 subdomain, which binds VEGF and supports semaphorin binding to the a1–a2 domain and the c domain, which mediates NRP dimerization. Semaphorin3A (SEMA3A), which has recently been reported to be a tumor suppressor gene, competitively binds to NRP1 to modulate VEGF–VEGFR–NRP1 interaction.

In the present study, to investigate the direct effect of VEGF on BC cells, we generated VEGF knocked-out MDA-MB-231 cells (231VEGFKO cells). Using these cells, we demonstrated that NRP1 signal by long isoform of VEGF (VEGF165), but not short isoform (VEGF121), regulates the morphology and migration ability of BC cells, and identified ARHGAP17, a rhoGAP, as a target gene of VEGF/NRP1 signaling. We also showed that VEGF/NRP1 signaling activates Cdc42 by suppressing ARHGAP17, and enhances filopodia formation and cell migration. Furthermore, using a publicly available dataset, we demonstrated that the VEGF/NRP1/ARHGAP17 regulatory network correlated with disease-free survival of hormone-receptor (HR)-negative BC patients.

Material and Methods
Detailed information regarding the material and methods used in this study is provided in the Supporting Information.

Cell culture and reagents
The cells and reagents used in this study are listed in Supporting Information.

CrisPR/Cas9 system and LentiCrisPRv2 infections
The CrisPR/Cas9 system was used to generate VEGF knockout MDA-MB-231 cells and Hs578T cells. The guide RNA (gRNA) sequences for knockdown of VEGFA were designed using a CrisPR gRNA Design Tool. The synthesized gRNA fragments were cloned into the BsmBI site of a lentivirus construct (Addgene, 52961), which contains a puromycin resistance marker. Viral particles containing gRNA for VEGFA and Cas9 were produced and transformed into MDA-MB-231 cells and Hs578T cells, according to the manufacturer’s instructions.

Development of MDA-MB-231 cells overexpressing soluble NRP1 (sNRP1)
The lentivirus system (Life Technologies, K2400-20, Carlsbad, CA) was used to introduce sNRP1 into wild-type MDA-MB-231 cells. Stably infected cells were selected with Blasticidin (10 μg/mL) for 7 days.

Migration assay
BC cells were placed into each Transwell® chamber and migrating cells were counted using an inverted microscope (Keyence, BZ-9000).

Knockdown of genes by siRNA oligos
SiRNA oligo duplexes, GeneSolution siRNA, were purchased from Qiagen (Germany). Information on the siRNAs is listed in Supporting Information Table S9. Cells were treated with complex of siRNA oligos (0.1–0.2 μg/well) and X-tremeGENE siRNA Transfection Reagent (Roche Diagnostics, Inc, Germany). Negative Control siRNAs (siControl, Qiagen) were used for mock transfection treatment.
Procedure to identify target genes of VEGF/NRP1 signal
To identify genes in the downstream of the VEGF/NRP1 signal network, we conducted genome-wide gene expression profiling of MDA-MB-231 cells (231WT, 231VEGF-KOex3 cells, and 231VEGF/KOex3 cells exposed to human recombinant VEGF165 (rhVEGF165). Agilent SurePrint G3 Human GE 8 x 60 K Microarray (Agilent technologies) was used. For the screening of differentially expressed genes, the microarray data was processed using Matlab software (R2016b, The Mathworks, Inc., MA).

G-LISA of Cdc42
To assess activation level of Cdc42, we used an ELISA-based assay, because G-LISA assay is more sensitive and quantitatively accurate than a GST–p21-activated kinase (PAK)–Cdc42-interactive binding domain pull-down assay as previously reported.25 The cells were seeded and serum starved by Cdc42-interactive binding domain pull-down assay as previously reported.25 The cells were seeded and serum starved by Cdc42-interactive binding domain pull-down assay as previously reported.25 The cells were seeded and serum starved by Cdc42-interactive binding domain pull-down assay as previously reported.25 The cells were seeded and serum starved by Cdc42-interactive binding domain pull-down assay as previously reported.25 The cells were seeded and serum starved by Cdc42-interactive binding domain pull-down assay as previously reported.25

Scanning electron microscopy
The cells were seeded onto polyethylene terephthalate coverslips (Thermanox XT™ disks, NUNC, Rochester, NY). The cells were serially fixed with 2% glutaraldehyde (Wako Pure Chemical Industries, Kyoto, Japan) at 4°C overnight, and then with 1% OsO4 for 2 hr. The fixed cells were dehydrated, dried, and coated with a thin layer of platinum palladium. The specimens were then scanned using an S-4700 scanning electron microscope (Hitachi, Tokyo, Japan). Then, for each cell, the number of filopodia of 50 cells were counted using ImageJ software. Protrusions which were thinner than 0.37 μm and longer than 0.87 μm were counted as filopodia.

Cell proliferation assay
We performed cell proliferation assay in two ways; direct counting and WST-8 assay (Cell Counting Kit-8, Dojindo Molecular Technologies, Inc., Kumamoto).

Perimeter measurement
Photo images for each cell were taken using a KEYENCE BZ-9000 microscope and the perimeters of the cells were measured using ImageJ software.

Validation analysis using a public dataset
The dataset of the METABRIC study was obtained from the cBioportal website. This dataset contains demographic information, survival outcomes, subtyping, gene-expression data and genomic abnormality. All survival analyses were performed using Matlab software (version 2016b).

Western blotting
To analyze ARHGAP17 and NRP1 protein level, we performed immunoblotting with anti-ARHGAP17 antibody (LSBio, Anti-ARHGAP17/NADRIN Antibody (aa331–380) LS-C120326, Seattle, WA) and anti-NRP1 antibody (Cell Signaling Technology, Neuropilin-1 (D62C6) Rabbit mAb, Danver, MA). Rather than using secondary antibody to probe for primary antibody binding, the Easy-Western-II detection system (Beacle, BCL-EZS21, Kyoto, Japan) was employed. For an internal control, we used mouse anti-β-actin antibody (Abcam, ab6276, Cambridge, UK, 1/5,000 dilution), and goat anti-mouse IgG antibody conjugated to a peroxidase (Pierce biotechnology, 31340, Rockford, IL, 1/50,000 dilution). The chemical luminescence reagent used was ECL Select (GE Healthcare, Buckinghamshire, UK). Signals were detected by Ez-CaptureII (ATTO, Tokyo, Japan) with ImageSaver5 software (ATTO).

Results
Knockout of VEGF gene altered cell morphology, and decreased migration ability of MDA-MB-231 cells
VEGF is a multifunctional molecule which targets a variety of cell members in the tumor microenvironment. The aim of the current study was to investigate the direct effects of VEGF on BC cells. First, we used bevacizumab for a loss-of-function experiment. According to qRT-PCR analysis of VEGF using a BC cell line panel, TNBC cell lines expressed higher level of VEGF than luminal BC cell lines (Supporting Information Figs. S1a and S1b). Among TNBC cells, we chose MDA-MB-231 (wild-type: 231WT) cells for the further experiments. A series of experiments showed that bevacizumab treatment did not induce any phenotypic change in cell proliferation (Fig. 1a), morphology (Figs. 2a, 2c and 2h) and migration (Fig. 2i) of 231WT cells.

Next, we knocked out VEGF of 231WT cells utilizing the CRISPR/Cas9 system to determine whether VEGF has direct effect on the phenotype of 231WT cells or not. VEGF has several transcriptional variants that correspond to different sizes of VEGF molecules, such as VEGF121, VEGF165, VEGF189, VEGF206 and others (Fig. 1b). We designed a gRNA sequence of CRISPR/Cas9 system to target exon3 of VEGF, that is common in all variant VEGF forms. Then, VEGF knocked-out MDA-MB-231 cells (231VEGF/KOex3) were established by introducing the gRNA and Cas9 using a lentiviral system. Then, genomic sequences of VEGF exon3 in VEGFKO cells were checked by Sanger Sequencing (Supporting Information Fig. S2a). Among seven 231VEGF/KOex3 clones checked, six
clones had frame-shift mutations that generate truncated VEGF molecules without any of VEGFR1- (exon3), VEGFR2- (exon4), heparin- (exon6–7) or neuropilin- (exon7) binding domains (Fig. 1b).26 In contrast, one clone had an in-frame mutation lacking only 12 amino acids. However, this deleted site overlapped with one of the dimerization sites (cysteine-notches) and a VEGFR1 binding site. This clone would not be functional. In addition, we confirmed by VEGF ELISA assay that the 231VEGFKOex3 cells did not actually secrete any detectable VEGF molecules (Fig. 1c).

Unlike the bevacizumab-treated 231WT cells, the 231VEGFKOex3 cells exhibited different phenotypes in cell morphology and migration from 231WT cells. The 231VEGFKOex3 cells had a small, rounded shape, whereas the 231WT cells showed a larger and spindle-like shape (Figs. 2a and 2b). To quantify the morphological features, we measured the perimeters of the different cell types using Image-J software. The perimeter of the 231VEGFKOex3 cells was significantly shorter than that of the 231WT cells (Fig. 2h, p < 0.0001). In addition, exposure of the 231VEGFKOex3 cells to exogenous rhVEGF165 restored their cell size, spindle-like shape (Fig. 2d), and perimeter to similar sizes, shapes and perimeter of those of the 231WT cells (Fig. 2h).

Next, cell migration assay using Transwell® chambers demonstrated that the 231VEGFKOex3 cells had lower migration ability than the 231WT cells (231WT vs. 231VEGFKOex3: p = 0.002), and that exposure to rhVEGF165 restored cell migration ability to an even higher level than the original level (231VEGFKOex3 vs. 231VEGFKOex3 + rhVEGF165: p = 0.0005) (Fig. 2i). These results indicate that VEGF depletion caused the morphologic change, and suppressed cell migration in the 231WT cells.

To eliminate the possibility of off-target effect of CRISPR-system, we also generated another VEGF knocked out 231 cell clone targeted at exon1 (231VEGFKOex1). Then, genomic sequences of VEGF exon1 in 231VEGFKOex1 cells were checked by Sanger Sequencing (Supporting Information Fig. 2b). The 231VEGFKOex1 cells had mostly similar morphological and cell motility phenotype to 231VEGFKOex3 cells. The 231VEGFKOex1 showed shorter perimeter (231WT vs. 231VEGFKOex1, p < 0.0001, Fig. 2h) and lower migration ability (231WT vs. 231VEGFKOex1, p = 0.0005, Fig. 2i) compared to 231WT cells.

These findings indicated that these morphological and cell motility phenotype is not due to off-target effect of CRISPR-system, but due to VEGF-depletion.
On the other hand, the knocking out of VEGF did not affect the cell proliferation of the 231<sup>WT</sup> cells (Fig. 1d). The doubling times of the 231<sup>WT</sup> and 231<sup>VEGFKOex3</sup> cells in the exponential phase (Day2–4) were almost identical at 21.7 and 21.2 hr, respectively. In summary, these data showed that knocking-out of VEGF induced rounded cell shape, and reduced cell migration, and indicated that VEGF could stimulate breast cancer cells directly.

Interaction between VEGF and NRP1 is responsible to phenotypic changes of 231<sup>VEGFKO</sup> cells

As the findings of the bevacizumab treatment and VEGF-knockout experiments appeared to differ, we endeavored to explain the underlying molecular mechanisms of these discrepancies. Bevacizumab is an anti-VEGF antibody that binds to the VEGFR1/2-binding domain (exon3–4) of VEGF to inhibit the VEGF/VEGFR signaling pathway. However, NRP1...
interacts with the exon7 portion of VEGF, which bevacizumab may be unable to block.27 Moreover, mRNA expression of VEGFR1/2, and NRP2 in the BC cells were significantly lower than those in the endothelial cells (Supporting Information Figs. S1c, S1d and S1g), whereas mRNA expression of NRP1 in the BC cells was comparable to that in the endothelial cells (Supporting Information Figs. S1e and S1f). Therefore, we hypothesized that signaling produced by VEGF/NRP1 without VEGFR1/2; or that the interaction inhibits a signal producing information Table S5). Although ARHGAP17 is not de
identified genes in downstream of VEGF/NRP1 signal pathway
To identify genes in the downstream of VEGF/NRP1 signal network, we conducted a microarray-based gene expression analysis of 231WT cells, 231VEGFKOex3 cells and 231VEGFKOex3 cells exposed to rhVEGF165. Genes in the downstream of the VEGF/NRP1 signals are supposed to be up/down-regulated in 231VEGFKOex3 cells, and rhVEGF165 treatment restores the expression to the 231WT cell level. Among the filtered expression data of 16,052 coding genes (Supporting Information Table S1), 765 genes were more than twofold up/down-regulated in the 231VEGFKOex3 cells (Fig. 3a, Supporting Information Table S2), compared to 231WT cells. Among these 765 genes, the expression levels of only 62 genes were restored by rhVEGF165 treatment (Fig. 3b). Thus, we chose these 62 genes as candidate genes (51 up-regulated and 11 down-regulated genes in 231VEGFKOex3 cells); they are listed in Supporting Information Table S3. To check whether this screening was properly executed, we performed gene ontology analysis using the DAVID tools.29,30 A functional annotation chart (Supporting Information Table S4) showed that, despite the 3rd rank in total, term "cell migration" was listed in the highest rank as bioprocess category, which would be compatible with our findings that VEGF/NRP1 signal is associated with cell migration.

ARHGAP17 (also known as RICH1) was up-regulated 4.6-fold in the 231VEGFKOex3 cells compared to the 231WT cells (Fig. 4a, 1.74-fold, p = 0.0036), and was restored by rhVEGF165 treatment (231VEGFKOex3 vs. 231VEGFKOex3 & rhVEGF165: 0.58-fold, p = 0.003). In addition, introduction of exogenous sNRP1 into the 231WT cells up-regulated ARHGAP17 expression (1.79-fold, p = 0.0029). Western blotting also showed ARHGAP17 expression was significantly up-regulated in the 231VEGFKOex3 cells compared to the 231WT cells by 2.14-fold (Fig. 4b).

The activity status of small GTPase molecules, including Cdc42, is regulated by GAPs, GEFs and GDIs. Among these 81 regulators, 11 ARHGAPs, 4 ARHGFEs and 2 GDIs are known to interact with Cdc42 and to inactivate it, a member of the Rho family, regulating cell cytoskeleton organization and cell migration. ARHGAP17 expression was validated by a quantitative RT-PCR method. ARHGAP17 expression was significantly up-regulated in the 231VEGFKOex3 cells compared to the 231WT cells by 2.14-fold (Fig. 4b).

Taken together, these results indicated that cell signals produced by VEGF–NRP1 interaction may contribute to phenotypic changes of 231VEGFKOex3 cells.
genes (Supporting Information Figs. S4b and S4c). Taken together, these microarray analysis results identified ARHGAP17 as a top candidate gene working in the downstream of VEGF/NRP1 signal, and we focused upon ARHGAP17 in further analysis.

Regulation of Cdc42 by ARHGAP17 is responsible for VEGF/NRP1-dependent morphologic and mobility changes

Rho family members, such as RhoA, Rac1 and Cdc42, cycle between inactive GDP-bound form and active GTP-bound form. ARHGAPs (RhoGAPs) hydrolyze GTP bound to active-
Figure 4. ARHGAP17 and Cdc42 status correlated with cell morphology and mobility. (a) mRNA expression of ARHGAP17 by qRT-PCR. (b) Western blotting shows ARHGAP17 was upregulated in 231VEGF KO3 cells compared to 231WT cells. It also shows ARHGAP17 expression was successfully knocked down by siRNA. (c) Quantitative measurement of active Cdc42 by G-LIZA assay. (d) Perimeters of siARHGAP17-treated cells, measured by Image-J. (e) Cell migration assay using Transwell chamber. (f) Count of filopodia per cell is shown. For each cell condition, the number of filopodia of 50 cells were counted. (g–n) Photos by scanning electron microscopy (g) 231WT cells, (h) 231VEGF KO3 cells, (i) rhVEGF165-treated 231VEGF KO3 cells, (j) bevacizumab treated MDA-MB-231 cells, (k) 231sNRP1 cells and (m) control cells. In a, c, and e, bars and error bars: mean ± standard deviation of triplicated experiments. In a and c–e, p: p-values of Student’s t-test, NS: not significant. [Color figure can be viewed at wileyonlinelibrary.com]
form into GDP and phosphate to inactivate Rho family members, whereas ARHGGEF (RhoGGEF) exchange GDP bound to inactive-form with GTP to activate the Rho family members. ARHGAP17 acts as a Cdc42-selective RhoGAP, and it inactivates Cdc42. As shown above, ARHGAP17 expression was regulated by VEGF/NRP1 signaling. Thus, to verify the relationship between VEGF/NRP1 signaling and Cdc42 status, we performed G-LISA assays on Cdc42 to measure and compare the amount of active Cdc42 in various cells as follows.

First, the association between ARHGAP17 and Cdc42 status was confirmed. In this analysis, we used two siRNAs to knock down ARHGAP17 of 231 cells. Introducing siRNAs against ARHGAP17 (Fig. 4b, Supporting Information Fig. S5) upregulated active Cdc42 level for more than twofold (Fig. 4c, 2.59-fold, p = 0.05), indicating that ARHGAP17 expression inversely correlated with Cdc42 status. Second, association between VEGF/NRP1 signaling and Cdc42 status was confirmed. The amounts of active Cdc42 were reduced only in the 231 cells and the 231 cells in which the ARHGAP17 level was up-regulated (p-value = 0.0016 and 0.006, respectively). In contrast, active Cdc42 levels were not reduced in the 231 cells treated with rhVEGF165 or the bevacizumab-treated 231 cells, in which the ARHGAP17 level was not altered (Fig. 4c).

Cdc42 activity is regulated by multiple GAPs, GEFs and GDIs. However, as shown in Figure 3c, only ARHGAP17 was altered by VEGF/NRP1 signaling. Thus, our findings implied that VEGF/NRP1 signaling regulates Cdc42 status via ARHGAP17.

To determine whether regulation of Cdc42 by ARHGAP17 is responsible for morphologic and mobility changes in 231 cells, restoration experiments of ARHGAP17 expression was performed by siARHGAP17. The knock down efficiencies of siARHGAP17 was checked by RT-PCR. The knock down efficiencies of siARHGAP17 oligos were 55, 41 and 59% for siARHGAP17–3, siARHGAP17–5, and both of them, respectively (Supporting Information Fig. S5). The knock down efficiency was also checked by western blotting. Protein level of ARHGAP17 was reduced by both siARHGAP17–3 and siARHGAP17–5 compared to siControl cells (Fig. 4b, 0.51-fold). Depending on the knock down efficiency of ARHGAP17, the perimeters and cell migration count of siRNA-treated 231 cells were recovered to the original level of the 231 cells (Figs. 4d and 4e).

These findings indicated that ARHGAP17-regulated Cdc42 is responsible for morphologic and mobility change in 231 cells.

VEGF/NRP1/ARHGAP17/Cdc42 network regulates filopodia formation in MDA-MB-231 cells

Cdc42 is crucial for the formation of filopodia, which is important in the motility of cancer cells. Thus, we hypothesized that filopodia formation induced by ARHGAP17/Cdc42 contributed to the VEGF/NRP1-regulated cell migration of 2913.
SEMA3A upregulation in 231\textsuperscript{VEGFKOex3} cells was due to a side effect of the CRISPR-Cas9 system.

**VEGF/NRP1/ARHGAP17 axis is not an uncommon regulatory network**

As above, we demonstrated that MDA-MB-231 cells had a VEGF/NRP1/ARHGAP17 regulatory network. To show generality of our findings, we produced a VEGF-null (exon3) Hs578T breast cancer cells (578\textsuperscript{VEGFKOex3} cells, Supporting Information Fig. S7). The 578\textsuperscript{VEGFKOex3} cells exhibited shorter perimeter morphology (Supporting Information Figs. S8a–S8c) and reduced cell migration ability (Supporting Information Fig. S8d). In addition, protein and mRNA expression of ARHGAP17 in 578\textsuperscript{VEGFKOex3} cells are upregulated compared to 578\textsuperscript{WT} cells (1.77-fold, Supporting Information Figs. S9a and S9b). These results are mostly identical to those of 231\textsuperscript{VEGFKOex3} cells. Therefore, the VEGF/NRP1/ARHGAP17 axis is not an unusual network.

**VEGF/NRP1-dependent regulation of ARHGAP17 in clinical samples**

Although the VEGF/NRP1/ARHGAP17/Cdc42 regulatory network works in one TNBC cell line (MDA-MB-231 cell), as demonstrated above, the prevalence of this network in clinical BC tissues remains unknown. To determine whether VEGF signal regulates ARHGAP17 expression in clinical BC tissue samples, we investigated the relationship between VEGF and ARHGAP17 using a publicly available dataset. The dataset of the METABRIC study\textsuperscript{36} was downloaded from the cBioportal website,\textsuperscript{37} which consisted of clinical information, copy number alteration (CNA) and gene expression data of 1,980 primary tumors of BC patients. Among the 1,980 BC samples, 1,468 had no CNA in NRP1 and ARHGAP17 locus. The expression data of VEGFA, SEMA3A and ARHGAP17 genes were extracted from the gene expression dataset. SEMA3A and VEGFA molecules competitively bind to the extracellular domain of NRP1 molecules;\textsuperscript{34} thus, distribution of ARHGAP17 and VEGFA/
SEMA3A ratio was shown in a scatter plot, as Supporting Information Figure S10a, and its correlation was estimated by Pearson’s and Spearman’s methods (Supporting Information Table S6). The samples were stratified into four subtypes according to conventional 3-gene classification (immunohistochemical expression of estrogen receptor, progesterone receptor and HER2). Expression of the ARHGAP17 and VEGFA/SEMA3A ratio was negatively correlated in pure HER2-type (n = 117) and TN subtypes (n = 263) with statistically significant p-values (Fig. 5n, p < 0.0001). In terms of prognosis, among the expression levels of VEGF, SEMA3A, NRP1 and ARHGAP17, the VEGF/SEMA3A expression ratio in the primary tumors of HR(−) BC significantly and inversely correlated with disease free survival (DFS) (logrank: p-value = 0.0244, Figs. 5b–5g, Supporting Information Table S7). Moreover, ARHGAP17 also tends to correlate with DFS.

Taken together, these findings suggest that the VEGF/NRP1/ARHGAP17 regulatory network would play a role in HR(−) BC tissues (Fig. 5e). Furthermore, scores of Cox’s proportional hazard model using VEGF/SEMA3A ratio and ARHGAP17 predict the DFS of patients efficiently (Fig. 5g). Thus, the VEGF/SEMA3A ratio and ARHGAP17 may be prognostic markers for HR(−) BCs.

Discussion
VEGF is secreted from a variety of cells in the tumor microenvironment, and plays key roles in tumor angiogenesis in collaboration with its receptors.5 Bevacizumab is a monoclonal antibody against VEGF that inhibits tumor angiogenesis and growth by blocking VEGF–VEGFR binding on endothelial cells, which is commonly used for the treatment of metastatic BC. To date, accumulating evidence has shown that VEGF is a multifunction molecule that affects a variety of cells in the tumor microenvironment, not only in endothelial cells.5 Thus, in the current study, we hypothesized that VEGF has a direct effect on BC cells that contributes to their malignant behavior. At first, in vitro experiments using bevacizumab did not show any phenotypic changes in MDA-MB-231 cells. This negative result would be reasonable because MDA-MB-231 cells do not express VEGFR1/2. To prove our hypothesis, we generated a VEGF-knocked out MDA-MB-231 (231VEGFKOex3) cells (Fig. 1). Unlike bevacizumab-treated MDA-MB-231 cells, 231VEGFKOex3 cells displayed morphologic and mobility changes (Fig. 2). Thus, we focused on NRP1 as another VEGF receptor.

The NRP1 consists of a large extracellular domain, one transmembrane domain and a relatively short cytoplasmic tail. In cases where VEGFR is expressed on the cell surface like endothelial cells, a VEGF dimer binds to the VEGFR/NRP1 complex. In such case, NRP1 acts as co-receptor, and the VEGF signal is introduced into cells through the cytoplasmic region of VEGFR. In the present study, we demonstrated that interaction between VEGF and NRP1 without VEGFR contributes to the phenotypic changes of MDA-MB-231 cells. However, how VEGF–NRP1 binding introduces signal into cells remains unknown. Two possible mechanisms for this are as follows. First, the short cytoplasmic tail of NRP1 does not have any tyrosine kinase domain, but a SEA motif that can bind PDZ domains of intracellular proteins such as GIPC (also known as synectin). GIPC is known to promote endothelial migration by collaborating with syndecan-4,38 and may be a candidate signal transduction molecule interacting with VEGF-activated NRP1. Another possible mechanism would be modulating the functions of other NRP1-interacting membrane receptors.

We demonstrated a function of the VEGF/NRP1/ARHGAP17 regulatory network in vitro. However, the function of this network in vivo had not yet been clarified. Previous studies showed that treatment using a NRP1 transmembrane domain interfering peptide inhibits the tumor growth and metastasis of xenograft of MDA-MB-231 cells.39 In addition, knock down of Cdc42-interacting protein-4 (CIP4) inhibited lung metastasis formation but not the tumor growth of MDA-MB-231 cells or HCC1806 cells.40 These data support our in vitro results.

The significance of the VEGF/NRP1/ARHGAP17 network in clinical samples was evaluated using a publicly available dataset: the METABRIC study. VEGF expression in clinical tissue specimens of BC is known to be related to patient outcome.6 In our analysis using the METABRIC study data (Supporting Information Figs. S10b–S10g), VEGF expression in the HR(+) cases significantly correlated with DFS, whereas NRP1 and ARHGAP17 expressions did not. In addition, the prognostic impacts (hazard ratios and p-values) of VEGF expression and VEGF/SEMA3A ratio were almost equal (Supporting Information Figs. 10b and 10f, Supporting Information Table S7), meaning that SEMA3A expression had almost no additional prognostic impact. Thus, VEGF/NRP1 signaling does not contribute to DFS in HR(+) BCs. In contrast, VEGF expression did not have significant impact to DFS in HR(−) BCs (Fig. 5g, p = 0.1398). However, ARHGAP17 expression tended to correlate with DFS (p = 0.0796), and the hazard ratio of the VEGF/SEMA3A ratio for DFS was higher than that of VEGF only. These results suggest that ARHGAP17 mediated by VEGF–NRP1 interaction partly contributed to DFS in HR(−) BCs. As shown in Supporting Information Table S10, the association between HR status and bevacizumab treatment efficacy in clinical trials has been inconsistent.41–47 Our findings demonstrated that there are bevacizumab-independent VEGF signals in HR(−) BC tumors. These data suggest that VEGF/NRP1/ARHGAP17 network-related markers could predict the outcomes of bevacizumab-related treatment in HR(−) BC cases, but not in HR(+) cases. A prospective study is required to prove the predictive values of these markers.

In this study, we showed that the knocking out of VEGF induced ARHGAP17 expression in MDA-MB-231 cells.
Several reports regarding ARHGAP17 focused on its function and interacting molecules. However, transcriptional regulation of ARHGAP17 remains unknown. Among BC subtypes, ARHGAP17 expression in HR(−) tumors is significantly higher than that in HR(+) tumors, according to the METABRIC dataset (Supporting Information Fig. S11). CNA in ARHGAP17 locus is relatively frequent for HR(+) BC but not for HR(−). Amplification in the ARHGAP17 locus was present in 26% of HR(+) BCs, but in just 2.6% of HR(−) cases (Chi-square test: $p < 0.0001$). In both the HR(−) and HR(+) tumors, the CNA status of the ARHGAP17 locus clearly correlated with ARHGAP17 expression. Thus, CNA is one of mechanisms that regulate ARHGAP17 expression, especially for HR(+) tumors. In terms of epigenetics, there is a CpG island extending from exon1 to intron1 of the ARHGAP17 gene. According to our previous methylation data of BC cell lines (GSE87177 in GEO database), the CpG island is mostly unmethylated in all BC cell lines. However, one probe at −290 bp from the transcriptional start site of ARHGAP17 was slightly differentially methylated between luminal (mean beta value: 0.319) and TN BC cell lines (mean beta value: 0.133) (Supporting Information Fig. S12). DNA methylation status around this area in the promoter region might affect transcription of ARHGAP17 especially for HR(+) tumors. Taken together, ARHGAP17 expression in HR(+) tumors is regulated by CNA and epigenetics, but CNA and epigenetics are not often involved in HR(−) tumors. Thus, VEGF/NRP1 signal in HR(−) tumors may regulate ARHGAP17 expression by other mechanisms, such as that which involves transcriptional factors.

To date, efforts have been made to find predictive blood-based biomarkers for bevacizumab treatment outcome, using samples collected in clinical trials. The AVADO trial for mBC and the AViTA trial for pancreatic cancer revealed that patients who express high baseline levels of short isoform VEGF exhibit improved progression-free survival and/or overall survival after bevacizumab treatment. These results have been explained as follows; an improved ELISA assay (modified IMPACT Elisa) used in these clinical trials could assess plasma VEGF level with greater sensitivity for short VEGF isoforms than the long isoforms. As shorter VEGF isoforms can diffuse over long distances, this assay could present the plasma level of tumor-derived VEGF that would be biologically relevant in the tumor microenvironment. Our results might provide another explanation for the predictive capability of the short VEGF isoform. Longer VEGF isoforms, such as VEGF165, can bind to both VEGFR and NRP1, whereas VEGF121 can bind to VEGFRs but not to NRP1. As shown in the current study, long VEGF isoforms (VEGF165) have

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**Figure 6.** Schematic representation outlining VEGF/NRP1/ARHGAP17/Cdc42 regulatory network in MDA-MB-231 cells. VEGF is secreted from cell members in tumor microenvironment, and acts as a multi-function molecule in autocrine and paracrine fashions. For endothelial cells, both short and long VEGF isoforms (such as VEGF121 and VEGF165) binds to VEGFR1/2 accompanied with NRP1, and promotes angiogenesis. In addition, bevacizumab can inhibit angiogenesis by blocking the interaction between both VEGF isoforms and VEGFR1/2. On the other hand, interaction between NRP1 and long VEGF isoform (VEGF165) but not short VEGF isoform (VEGF121) produces a signal into MDA-MB-231 cells to downregulate ARHGAP17 expression. The downregulation of ARHGAP17 activates Cdc42 status, and increases filopodia formation, resulting in enhanced cell migration ability. Because 231 cells do not express VEGFR1/2, bevacizumab cannot block this VEGF–NRP1 interaction. [Color figure can be viewed at wileyonlinelibrary.com]
high affinity to NRPI to produce signals modulating ARHGAP17. However, short VEGF isoforms (VEGF121) could not produce the signals. In short VEGF-dominant tumors, VEGF/VEGFR signaling would be dominant and could be efficiently blocked by bevacizumab. However, because not only VEGF/VEGFR signaling, but also VEGF/NRP1 signaling may work in long VEGF-dominant tumors, VEGF/NRP1 signaling could remain and contribute to malignant phenotypes of BC even during bevacizumab treatment. This might be a reason why bevacizumab treatment seems to yield better results in patients with high levels of short VEGF isoform.

In conclusion, this study demonstrated the existence of a novel VEGF-related regulatory network that is independent from bevacizumab-treatment. In MDA-MB-231 cells, The NRP1 signal produced by long isoform of VEGF reduces ARHGAP17 expression, and activates Cdc42. Then, Cdc42-mediated filopodia formation consequently increases cell migration (Fig. 6). In addition, the inverse correlation between VEGF and ARHGAP17 was relevant in clinical tissues, and the VEGF-ARHGAP17 network correlated with the prognosis of HR(−) BCs. Thus, the VEGF/NRP1/ARHGAP17/Cdc42 network can be a potential mechanism for bevacizumab treatment resistance.

**Author Contributions**

FS conceived the study, and MK, SS, MT, FS participated in its design and manuscript preparation. MK and ST carried out all wet lab experiments. FS and MK interpreted the data. FS performed bioinformatics and statistical analyses. All authors have read and approved the final manuscript.

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**References**

1. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-tieulent J, Jemal A. Global cancer statistics, 2012. CA Cancer J Clin [Internet] 2015;65:87–108. Available from: http://onlinelibrary.wiley.com/doi/10.3322/caac.21262/abstract

2. Gierz GL, Burke A, Anderson WF. Epidemiology of triple negative breast cancers. Breast Dis [Internet] 2010;32:5–24. Available from: http://www.nature.com/doifinder/10.1007/s10689-009-9214-0; http://www.medra.org/servlet/articleResolver; http://www.ncbi.nlm.nih.gov/pubmed/21965309

3. Weigelt B, Peterse JL, van’t Veer LJ. Breast cancer metastasis: markers and models. Nat Rev Cancer 2005;5:591–602.

4. Wan L, Pantel K, Kang Y. Tumor metastasis: moving new biological insights into the clinic. Nat Med [Internet] 2013;19:1450–64. Available from: http://www.nature.com.proxy.library.adelaide.edu.au/nm/journal/v19/n11/full/nm.3391.html

5. Godd H, Mercurio AM. VEGF targets the tumour cell. Nat Rev Cancer [Internet] 2013;13:871–2. Available from: http://doi.org/10.1038/nrc3627

6. Gasparini G, Toi M, Gion M, et al. Prognostic significance of vascular endothelial growth factor protein in node-negative breast carcinoma. J Natl Cancer Inst 1997;89:139–47.

7. Schneider BP, Wang M, Radovich M, et al. Association of vascular endothelial growth factor receptor-2 genetic polymorphisms with outcome in a trial of paclitaxel compared with paclitaxel plus bevacizumab in advanced breast cancer: ECOCG 2100. J Clin Oncol 2008;26:4672–8.

8. Miles DW, Chan A, Dirix LY, et al. Phase III study of bevacizumab plus docetaxel compared with placebo plus docetaxel for the first-line treatment of human epidermal growth factor receptor 2-negative metastatic breast cancer. J Clin Oncol 2010;28:3239–47.

9. Robert NJ, Dieras V, Glaspy J, et al. RIBBON-1: randomized, double-blind, placebo-controlled, phase III trial of chemotherapy with or without bevacizumab for first-line treatment of human epidermal growth factor receptor 2-negative, locally recurrent or metastatic breast cancer. J Clin Oncol 2011;29:1252–60.

10. Orimo A, Gupta PB, Sgroi DC, et al. Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. Cell 2005;121:335–48.

11. Casanova O, Hicklin DJ, Bergers G, et al. Drug resistance by evasion of antiangiogenic targeting of VEGF signaling in late-stage pancreatic ductal adenocarcinoma. Cancer Cell 2005;8:299–309.

12. Crawford Y, Kamsan I, Yu L, et al. PDGF-C mediates the angiogenic and tumorigenic properties of fibroblasts associated with tumors refractory to anti-VEGF treatment. Cancer Cell 2009;15:21–34.

13. Leite de Oliveira R, Hamm A, Mazzone M. Growing tumor vessels: more than one way to skin a cat - Implications for angiogenesis targeted cancer therapies. Mol Aspects Med 2011;32:71–87.

14. Lyden D, Hattori K, Diao S, et al. Impaired recruitment of bone-marrow-derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth. Nat Med 2001;7:1194–201.

15. Shojaei F, Wu X, Malik AK, et al. Tumor refractoriness to anti-VEGF treatment is mediated by its ability to produce signals modulating ARHGAP17. However, short VEGF isoforms (VEGF121) could not produce the signals. In short VEGF-dominant tumors, VEGF/VEGFR signaling would be dominant and could be efficiently blocked by bevacizumab. However, because not only VEGF/VEGFR signaling, but also VEGF/NRP1 signaling may work in long VEGF-dominant tumors, VEGF/NRP1 signaling could remain and contribute to malignant phenotypes of BC even during bevacizumab treatment. This might be a reason why bevacizumab treatment seems to yield better results in patients with high levels of short VEGF isoform.

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**References**

1. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-tieulent J, Jemal A. Global cancer statistics, 2012. CA Cancer J Clin [Internet] 2015;65:87–108. Available from: http://onlinelibrary.wiley.com/doi/10.3322/caac.21262/abstract

2. Gierz GL, Burke A, Anderson WF. Epidemiology of triple negative breast cancers. Breast Dis [Internet] 2010;32:5–24. Available from: http://www.nature.com/doifinder/10.1007/s10689-009-9214-0; http://www.medra.org/servlet/articleResolver; http://www.ncbi.nlm.nih.gov/pubmed/21965309

3. Weigelt B, Peterse JL, van’t Veer LJ. Breast cancer metastasis: markers and models. Nat Rev Cancer 2005;5:591–602.

4. Wan L, Pantel K, Kang Y. Tumor metastasis: moving new biological insights into the clinic. Nat Med [Internet] 2013;19:1450–64. Available from: http://www.nature.com.proxy.library.adelaide.edu.au/nm/journal/v19/n11/full/nm.3391.html

5. Godd H, Mercurio AM. VEGF targets the tumour cell. Nat Rev Cancer [Internet] 2013;13:871–2. Available from: http://doi.org/10.1038/nrc3627

6. Gasparini G, Toi M, Gion M, et al. Prognostic significance of vascular endothelial growth factor protein in node-negative breast carcinoma. J Natl Cancer Inst 1997;89:139–47.

7. Schneider BP, Wang M, Radovich M, et al. Association of vascular endothelial growth factor receptor-2 genetic polymorphisms with outcome in a trial of paclitaxel compared with paclitaxel plus bevacizumab in advanced breast cancer: ECOCG 2100. J Clin Oncol 2008;26:4672–8.

8. Miles DW, Chan A, Dirix LY, et al. Phase III study of bevacizumab plus docetaxel compared with placebo plus docetaxel for the first-line treatment of human epidermal growth factor receptor 2-negative metastatic breast cancer. J Clin Oncol 2010;28:3239–47.

9. Robert NJ, Dieras V, Glaspy J, et al. RIBBON-1: randomized, double-blind, placebo-controlled,
28. Gagnon ML, Bielenberg DR, Gechzman Z, Miao HQ, Takashima S, Soker S, Klagesrn M. Identification of a natural soluble neuropilin-1 that binds vascular endothelial growth factor: in vivo expression and antitumor activity. Proc Natl Acad Sci U S A [Internet] 2000;97:2573–8. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=15970&tool=pmc&rendertype=abstract

29. Huang DW, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc 2009;4:44–57.

30. NIH. DAVID bioinformatics resources 6.8 [Internet]. Available from: https://david.ncifcrf.gov

31. Wells CD, Fawcett JP, Traweger A, et al. Identification of a natural soluble neuropilin-1 regulating the alpha2β1 integrin activity. J Transl Med 2013;11:1–16.

32. Pan H, Wanami LS, Disanayake TR, et al. Autocrine semaphorin3A stimulates alpha2 beta1 integrin expression/function in breast tumor cells. Breast Cancer Res Treat 2009;118:197–205.

33. Bear HD, Tang G, Rastogi P, Geyer Jr. CE, Robidoux A, Atkins JN, Baez-Diaz L, Brutsky AM, Mehta RS, Febrhenbacher L, Young JA, Senecal FM, et al. Bevacizumab added to neoadjuvant chemotherapy for breast cancer. N Engl J Med [Internet] 2012;366:310–20. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22276821

35. Bachelder RE, Crago A, Jung J, et al. Advances in brief vascular endothelial growth factor is an autocrine survival factor for neuropilin-expressing breast carcinoma cells 1. Cancer Res 2001;61:5736–40.

36. Pereira B, Chin S-F, Ruenda OM, Vollan H-KM, Provenzano E, Bardwell A, Pugh M, Jones L, Russell R, Sammut S-J, Tsui DWY, Liu B, et al. The somatic mutation profiles of breast cancers reflect their genomic and transcriptomic landscapes. Nat Commun [Internet] 2016;7:11479. Available from: http://www.nature.com/doifinder/10.1038/ncomms11479

37. eBioPortal for Cancer Genomics [Internet]. Available from: http://www.ebioportal.org/study?id=brca_metabric#summary

39. Arpel A, Gamper C, Spělčí C, et al. Inhibition of primary breast tumor growth and metastasis using a neuropilin-1 transmembrane domain interfering peptide. Oncotarget 2016;7:54723–32.

42. Vivot X, Schneeveis A, Verma S, et al. Efficacy and safety of bevacizumab in combination with docetaxel for the first-line treatment of elderly patients with locally recurrent or metastatic breast cancer: results from AVADO. Eur J Cancer 2011;47:2387–95.

43. von Minckwitz G, Eidtmann H, Rezai M, von Minckwitz G, Puglisi F, Cortes J, Vrdoljak E, Romieu GH, Lichinitser M, Rastogi P, et al. AVEREL: a randomized phase III Trial evaluating bevacizumab in combination with docetaxel and trastuzumab as first-line therapy for HER2-positive locally recurrent/metastatic breast cancer. J Clin Oncol 2013;31:1719–25.

44. Gianni L, Romieu GH, Lichinitser M, et al. AVEREL: a randomized phase III Trial evaluating bevacizumab in combination with docetaxel and trastuzumab as first-line therapy for HER2-positive locally recurrent/metastatic breast cancer. J Clin Oncol 2013;31:1719–25.

45. Kobayashi Y, Harada A, Furuta B, et al. The role of NADRIN , a Rho GT-Pase-activating protein , in the morphological differentiation of astrocytes. J Biochem 2013;153:389–98.

46. Bear HD, Tang G, Rastogi P, et al. Bevacizumab added to neoadjuvant chemotherapy for breast cancer. N Engl J Med [Internet] 2012;366:299–309.

47. Earl HM, Hiller L, Dunn JA, Blenkinsop C, Grybovicki L, Vallier AL, Abraham J, Thomas J, Provenzano E, Hughes-Davies L, Gounaris I, McAdam K, Chan S, Ahmad R, Hichki T, Houston S, Rea D, Bartlett J, Caldas C, Cameron DA, Hayward L, ARTemis Investigators. Efficacy of neoadjuvant bevacizumab added to docetaxel followed by fluorouracil, epirubicin, and cyclophosphamide, for women with HER2-negative early breast cancer (ARTemis): an open-label, randomised, phase 3 trial. Lancet Oncol 2015;16:e56–66. Available from: https://doi.org/10.1016/S1470-2045(15)70137-3

48. Kobayashi Y, Harada A, Furuta B, et al. The role of NADRIN , a Rho GT-Pase-activating protein , in the morphological differentiation of astrocytes. J Biochem 2013;153:389–98.