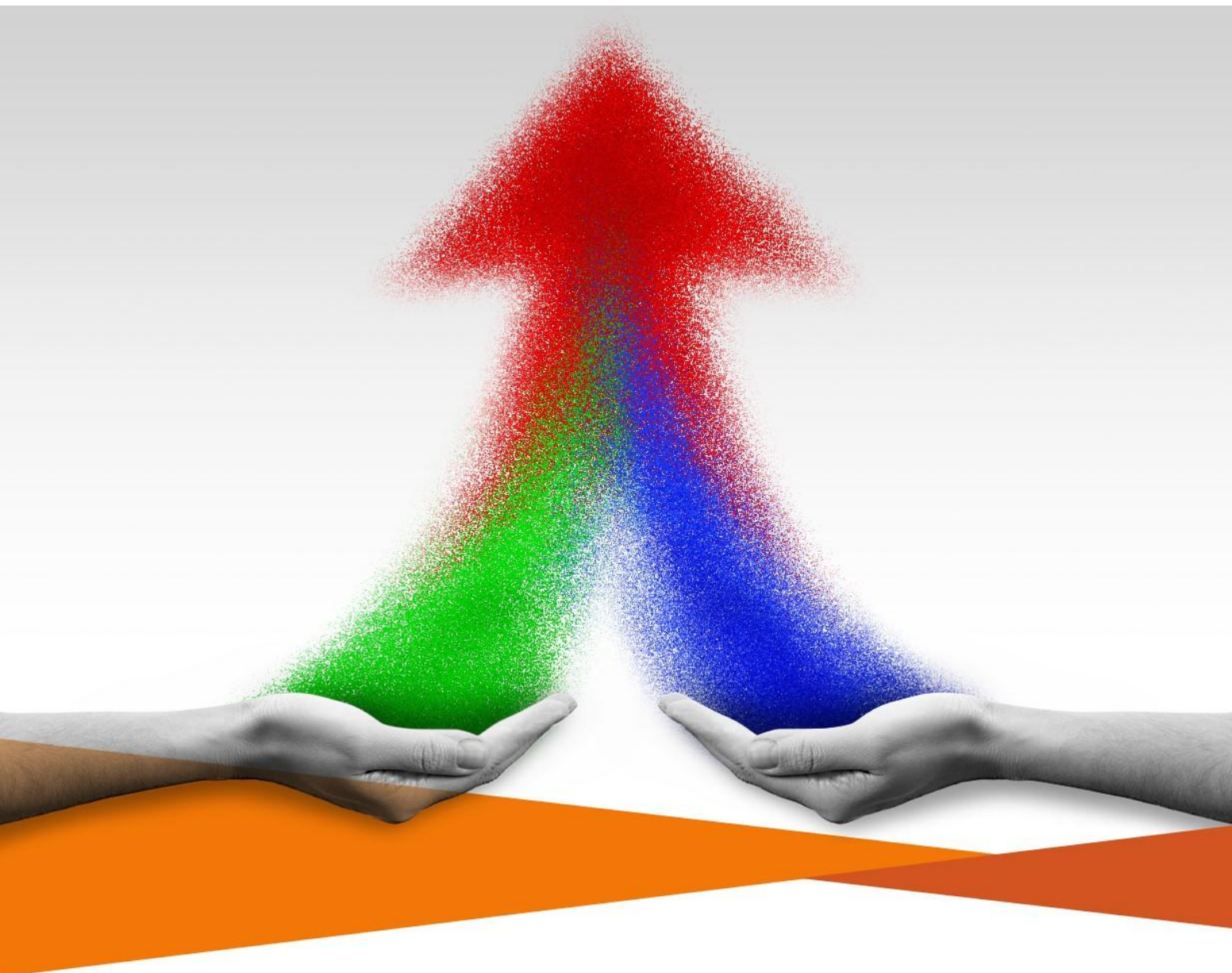




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Long isoform of VEGF stimulates cell migration of breast cancer by filopodia formation via NRP1/ARHGAP17/Cdc42 regulatory network

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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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Novelty and Impact: VEGF stimulates endothelial cells as an angiogenic molecule. However, effects of VEGF on breast cancer (BC) cells are not well understood. Here, we found that long isoform of VEGF (VEGF165) can generate a signal through NRP1 without VEGFR into BC cells. In addition, we identified a target molecule of this signal, ARHGAP17, that regulates Cdc42 activity and controls filopodia formation and cell migration ability. This network may contribute to malignant behaviors of hormone-receptor negative BC.

Abstract

VEGF stimulates endothelial cells as a key molecule in angiogenesis. VEGF also works as a multi-function 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 (231^{VEGFKOex3}) showed rounded morphology and shorter perimeter (1.6-fold, $P<0.0001$). 231^{VEGFKOex3} 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 (231^{sNRP1}) exhibited rounded morphology and shorter perimeter (1.3-fold, $P<0.0001$). The 231^{sNRP1} cells also showed impaired cell migration (1.7-fold, $P=0.003$). These changes were similar to that of 231^{VEGFKOex3} 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*. According to the database of 1,980 breast cancer cases, the ratio of *VEGF* and *SEMA3A* in primary tumors 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.

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 worldwide¹. 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 prognosis². Approximately 15% of BC patients develop distant metastasis, which is responsible for about 90% of BC-associated mortality³. 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 environments⁴. 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 microenvironment⁵. 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 prognosis⁶. Bevacizumab, an antibody that neutralizes VEGF, suppresses tumor angiogenesis by blocking the VEGF signals in endothelial cells. The Eastern Cooperative Oncology Group (ECOG) conducted a randomized trial (E2100 trial) for metastatic BC patients to compare treatment arms of paclitaxel plus bevacizumab and paclitaxel alone⁷. The bevacizumab-containing regimen resulted in a significantly

improved progression-free survival (PFS) (11.8 vs 5.9 months, hazard ratio: 0.60, $P < 0.001$), although it did not have a significant impact on overall survival (OS). Two other bevacizumab-related randomized trials for metastatic BC, the AVADO trial⁸ and the RIBBON-1 trial⁹, confirmed the E2100's findings that bevacizumab prolonged PFS but not OS.

Several mechanisms of this acquired resistance have been proposed, such as angiogenesis by alternative pro-angiogenic pathways¹⁰⁻¹³, and recruitment of vascular progenitors¹⁴ and modulators^{15,16}. In addition, hypoxia induced by anti-angiogenic therapy promotes selection of aggressive cancer cells¹⁷ and expansion of cancer stem cell pool¹⁸. According to recent studies, VEGF is a multi-function molecule targeting not only endothelial cells but also other cell members in the tumor microenvironment, including cancer cells, fibroblasts, immune cells, and more⁵. However, VEGF's direct effect on cancer cells is not yet fully understood. Thus, we focused on the direct effect of VEGF on cancer cells, and hypothesized that there would be a VEGF-induced signal that contributes to the malignant behavior of BC cells.

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-1/2¹⁹⁻²². 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 dimerization²³. Semaphorin3A (SEMA3A), which has recently been

reported to be a tumor suppressor gene²⁴, competitively binds to NRP1 to modulate VEGF-VEGFR-NRP1 interaction²³.

In the present study, to investigate the direct effect of VEGF on BC cells, we generated VEGF knocked-out MDA-MB-231 cells (231^{VEGFKO} 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 Supplemental Information.

Cell Culture and Reagents

The cells and reagents used in this study are listed in Supplemental 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 lentiCRISPRv2 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, USA) was used to introduce sNRP1 into wild-type MDA-MB-231 cells. Stably infected cells were selected with Blasticidin (10 µg/mL) for seven 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 Supplementary Table 9. Cells were treated with complex of siRNA oligos (0.1-0.2µg/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 the target genes in the downstream of the VEGF/NRP1 signal network, we conducted genome-wide gene expression profiling of MDA-MB-231 cells (231^{WT}), 231^{VEGFKOex3} cells and 231^{VEGFKOex3} cells exposed to human recombinant VEGF165 (rhVEGF165). Agilent SurePrint G3 Human GE 8 × 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, USA).

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 reported²⁵. The cells were seeded and serum starved by serial incubations with 10% FBS for 72 hours, 0.5% FBS for 24 hours, and then 0% FBS for 24 hours. Cdc42 activation level was measured using a G-LISA kit for Cdc42 (colorimetric format; Cytoskeleton, Denver, CO, USA) according to the manufacturer's protocol. Developed color was scanned by a microplate reader (SPECTRAMAX340PC, Molecular Devices, CA, USA).

ELISA for VEGF

VEGF concentration in culture media was analyzed using a colorimetric ELISA kit for human VEGF (Cat. No.RSD-DVE00-1, R&D Systems Inc.).

Scanning Electron Microscopy

The cells were seeded onto polyethylene terephthalate coverslips (Thermanox X™ disks, NUNC, Rochester, NY, USA). 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 hours. 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 Tm 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, USA) and anti-NRP1 antibody (Cell Signaling Technology, Neuropilin-1 (D62C6) Rabbit mAb, Danver, MA, USA). 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, 1/5000 dilution), and goat anti-mouse IgG antibody conjugated to a peroxidase (Pierce biotechnology, 31340, Rockford, IL, USA, 1/50000 dilution). The chemical luminescence reagent used was ECL Select (GE Healthcare, Buckinghamshire, UK). Signals were detected by Ez-Capture II (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 multi-functional 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 (Supplementary Fig. 1a, b). Among TNBC cells, we chose MDA-MB-231 (wild-type: 231^{WT}) cells for the further experiments. A series of experiments showed that bevacizumab treatment didn't induce any phenotypic change in cell proliferation (Fig. 1a), morphology (Fig. 2a, 2c, and 2h), and migration (Fig. 2i) of 231^{WT} cells.

Next, we knocked out *VEGF* of 231^{WT} cells utilizing the CRISPR/Cas9 system to determine whether VEGF has direct effect on the phenotype of 231^{WT} 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 (231^{VEGFKOex3}) 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 (Supplementary Fig. 2-a). Among seven 231^{VEGFKOex3} 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 twelve 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 231^{VEGFKOex3} cells did not actually secrete any detectable VEGF molecules (Fig. 1c).

Unlike the bevacizumab-treated 231^{WT} cells, the 231^{VEGFKOex3} cells exhibited different phenotypes in cell morphology and migration from 231^{WT} cells. The 231^{VEGFKOex3} cells had a small, rounded shape, whereas the 231^{WT} cells showed a larger and spindle-like shape (Fig. 2a-b). To quantify the morphological features, we measured the perimeters of the different cell types using Image-J software. The perimeter of the 231^{VEGFKOex3} cells was significantly shorter than that of the 231^{WT} cells (Fig. 2h, $P < 0.0001$). In addition, exposure of the 231^{VEGFKOex3} 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 231^{WT} cells (Fig. 2h).

Next, cell migration assay using Transwell® chambers demonstrated that the 231^{VEGFKOex3} cells had lower migration ability than the 231^{WT} cells (231^{WT} vs 231^{VEGFKOex3}: $P = 0.002$), and that exposure to rhVEGF165 restored cell migration ability to an even higher level than the original level (231^{VEGFKOex3} vs 231^{VEGFKOex3}+rhVEGF165: $P = 0.0005$) (Fig. 2i). These results indicate that VEGF depletion caused the morphologic change, and suppressed cell migration in the 231^{WT} 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 ($231^{VEGFKOex1}$). Then, genomic sequences of VEGF exon1 in $231^{VEGFKOex1}$ cells were checked by Sanger Sequencing (Supplementary Fig. 2-b). The $231^{VEGFKOex1}$ cells had mostly similar morphological and cell motility phenotype to $231^{VEGFKOex3}$ cells. The $231^{VEGFKOex1}$ showed shorter perimeter (231^{WT} vs $231^{VEGFKOex1}$ $P < 0.0001$, Fig.2h) and lower migration ability (231^{WT} vs $231^{VEGFKOex1}$ $P = 0.0005$, Fig.2i) compared to 231^{WT} 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^{WT} cells (Fig. 1d). The doubling times of the 231^{WT} and $231^{VEGFKOex3}$ cells in the exponential phase (Day2-4) were almost identical at 21.7 and 21.2 hours, 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^{VEGFKO} 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 block²⁷. Moreover, mRNA expression of VEGFR1/2, and NRP2 in the BC cells were significantly lower than those in the endothelial cells (Supplementary Fig. 1c, d, and g), whereas mRNA expression of NRP1 in the BC cells was comparable to that in the endothelial cells (Supplementary Fig. 1e, f). Therefore, we hypothesized that signaling produced by VEGF/NRP1 without VEGFR1/2 may contribute to the phenotypic changes of VEGF-Knocked out cells.

To prove the hypothesis, we utilized a soluble form of neuropilin-1 (sNRP1), a naturally-existing isoform of NRP1, which consists of the NRP1 extracellular domain with a VEGF-binding site. The sNRP1 works as a VEGF-Trap to inhibit the VEGF-NRP1 interaction²⁸. Then, sNRP1 was introduced into the MDA-MB-231 cells (231^{sNRP1} cells) by a lentivirus vector system. As expected, the 231^{sNRP1} cells showed smaller and more rounded morphology (Fig. 2f), with a significantly shorter perimeter than the control cells (Fig. 2h, 1.3-fold, $P < 0.0001$). In addition, the 231^{sNRP1} cells had lower migration ability than the control cells (Fig. 2i, 1.7-fold, $P = 0.003$). These findings demonstrated that the phenotypes of the 231^{sNRP1} cells were similar to those of the 231^{VEGFKOex3} cells, and indicate that interaction of VEGF and NRP1 contribute to phenotypic changes of cell morphology and motility. However, it remains unknown as to which of the following two scenarios are true: that the VEGF-NRP1 interaction directly produces a signal without VEGFR1/2; or that the interaction inhibits a signal produced by NRP1 and another NRP1-interacting molecule, such as SEMA3A. To determine whether VEGF-NRP1 interaction directly produces a signal or not, NRP1 expression was knocked down by siRNA (Fig 2g, Supplementary Fig. 3). The knock down efficiencies of siNRP1 was checked by

RT-PCR blotting and western blotting. The knock down efficiencies of siNRP1-7 and siNR1-12 were 54.2% and 58.9% in mRNA level, respectively (Supplementary Fig.3). The knock down efficiencies were 24.9% and 61.1% for protein level of membrane anchored NRP, and 10.8% and 76.6% for sNRP1, respectively (Fig.2g). The NRP1-knocked down 231 (231^{NRP1KD}) cells exhibited lower cell migration ability than the 231^{WT} cells (Fig. 2i, 3.7-fold, $P=0.017$). Assuming SEMA3A-NRP1 binding produce a signal to induce rounded cell shape and to reduce cell migration in $231^{VEGFKOex3}$ cells, 231^{sNRP1} cells and 231^{NRP1KD} cells would lose this SEMA3A-NRP1 signal and are supposed to make different cell morphology and mobility. However, actual morphologic and mobility phenotypes of $231^{VEGFKOex3}$ cells, 231^{sNRP1} cells, and 231^{NRP1KD} cells displayed almost identical phenotypes. Thus, we concluded that VEGF-NRP1 binding but not SEMA3A-NRP1 binding produced a signal to regulate cell morphology and cell migration ability.

Taken together, these results indicated that cell signals produced by VEGF-NRP1 interaction may contribute to phenotypic changes of $231^{VEGFKOex3}$ cells.

Identification of 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 231^{WT} cells, $231^{VEGFKOex3}$ cells, and $231^{VEGFKOex3}$ cells exposed to rhVEGF165. Genes in the downstream of the VEGF/NRP1 signals are supposed to be up/down-regulated in $231^{VEGFKOex3}$ cells, and rhVEGF165 treatment restores the expression to the 231^{WT} cell level. Among the filtered expression data of 16052 coding genes

(Supplementary Table 1), 765 genes were more than 2-fold up/down-regulated in the 231^{VEGFKOex3} cells (Fig. 3a, Supplementary Table 2), compared with 231^{WT} 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 231^{VEGFKOex3} cells); they are listed in Supplementary Table 3. To check whether this screening was properly executed, we performed gene ontology analysis using the DAVID tools^{29,30}. A functional annotation chart (Supplementary Table 4) 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- ($=2^{2.212}$) fold in the 231^{VEGFKOex3} cells (Fig. 3c, Supplementary Table 3). Although ARHGAP17 is not defined as a cell migration-related gene in the DAVID files, it is a RhoGAP that is known to interact with Cdc42³¹ 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 231^{VEGFKOex3} cells compared with the 231^{WT} cells (Fig. 4a, 1.74-fold, $P=0.0036$), and was restored by rhVEGF165 treatment (231^{VEGFKOex3} vs 231^{VEGFKOex3}+rhVEGF165: 0.58-fold, $P=0.003$). In addition, introduction of exogenous sNRP1 into the 231^{WT} cells up-regulated ARHGAP17 expression (1.79-fold, $P=0.0029$). Western blotting also showed ARHGAP17 expression was significantly up-regulated in the 231^{VEGFKOex3} cells compared to the 231^{WT} 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 ARHGEFs, and 2 GDIs are known to interact with Cdc42 (Supplementary Table 5a). Among 17 Cdc42-regulators, only *ARHGAP17* meet selection criteria as VEGF-dependent genes (Fig. 3c, Supplementary Fig. 4a). As demonstrated in previous reports, ARHGAP17 interacts with Cdc42 and regulates its activeness³¹. Similarly, the expression levels of 42 genes that are known to interact with NRP1 were also checked (Supplementary Table 5b). However, knocking out VEGF did not alter the expression of any NRP1-interacting genes (Supplementary Fig. 4b-c). 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/NRP-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-form into GDP and phosphate to inactivate Rho family members, whereas ARHGEFs (RhoGEPs) exchange GDP bound to inactive-form with GTP to activate the Rho family members. ARHGAP17 acts as a Cdc42-selective RhoGAP, and it inactivates Cdc42³¹. 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^{VEGFKOex3} cells. Introducing siRNAs against ARHGAP17 (Fig.4b, Supplementary Fig. 5) upregulated active Cdc42 level for more than 2-fold (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^{VEGFKOex3} cells and the 231^{sNRP1} 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^{VEGFKOex3} cells treated with rhVEGF165 or the bevacizumab-treated 231^{WT} 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 Fig. 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^{VEGFKOex3} 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 (Supplementary Fig. 5). 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^{VEGFKOex3} cells were recovered to the original level of the 231^{WT} cells (Fig. 4d-e).

These findings indicated that ARHGAP17-regulated Cdc42 is responsible for morphologic and mobility change in 231^{VEGFKOex3} 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 cells³². Thus, we hypothesized that filopodia formation induced by ARHGAP17/Cdc42 contributed to the VEGF/NRP1-regulated cell migration of MDA-MB-231 cells. We used a scanning electron microscope to observe the filopodia formation of cells. We also counted filopodia number of each cells (Fig.4f). Most of the spreading 231^{WT} cells displayed extended filopodia (Fig. 4g), whereas the 231^{VEGFKOex3} cells had significantly fewer filopodia (Fig. 4h, $P<0.0001$). In addition, the extension of filopodia was restored by rhVEGF165 treatment to the 231^{VEGFKOex3} cells (Fig. 4i, $P<0.0001$). Furthermore, bevacizumab treatment did not show any decrease in filopodia formation (Fig.4k, $P=0.1521$), whereas sNRP1 overexpression caused decrease of filopodia formation compared to control cells (Fig.4m-n, $P<0.0001$). Thus, for the MDA-MB-231 cells, filopodia formation was associated with VEGF signal and Cdc42 activation, and reduction of filopodia formation was correlated with upregulation of ARHGAP17 and inhibition of VEGF.

Taken together, our findings indicate that VEGF/NRP1 signaling regulates cell migration by filopodia formation via ARHGAP17/Cdc42 in MDA-MB-231 cells.

VEGF isoforms differentially regulate ARHGAP17

As shown in Fig.1b, VEGF has various isoforms with different sizes. In experiments above, we used recombinant protein of the most dominant isoform, VEGF165, that contains a neuropilin-binding domain corresponding to exon7-8a. A short isoform, VEGF121, lacks a part of neuropilin binding domain corresponding to exon7, but still has six amino acid tail of exon8a that can bind to NRP1. To determine whether these different VEGF isoforms regulate ARHGAP17 or not, we treated 231^{VEGFKOex3} cells with rhVEGF121. As shown in Fig. 2h, 2i, 4f, and 4j, for the 231^{WT} cells, the rhVEGF165 restored cell perimeter, cell migration, and filopodia formation to 231^{WT} cell level, whereas rhVEGF121 could not affect it. These results support that interaction between NRP1 and NRP-binding domain of VEGF molecule, corresponding to exon7, is responsible to regulating ARHGAP17, and phenotypic changes of 231^{VEGFKOex3} cells.

Semaphorin 3A was not regulated by VEGF/NRP1 signal.

Semaphorin 3A (SEMA3A) is a member of the semaphorin family that plays a critical role in axonal guidance, and that has recently been recognized as a tumor suppressor^{24,33}. SEMA3A and VEGF interact with the b1 domain of NRP1³⁴. Thus, SEMA3A can competitively block the binding of VEGF to NRP1. In a previous study, the VEGF/SEMA3A protein ratio was correlated with the cell migration of the BC cell lines³⁵. Therefore, *SEMA3A* expression should be monitored concurrently with VEGF expression. In this study, *SEMA3A* mRNA level was upregulated in the 231^{VEGFKOex3} cells compared to the 231^{WT} cells

(Supplementary Fig. 6). However, the *SEMA3A* level in the 231^{VEGFKOex3} cells was not restored to the original level by the rhVEGF165 treatment. In addition, introduction of exogenous sNRP1 to the 231^{WT} cells did not alter the *SEMA3A* level. Taken together, *SEMA3A* expression was not controlled by VEGF/NRP1 signaling, and *SEMA3A* upregulation in 231^{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-knocked out (exon3) Hs578T breast cancer cells (578^{VEGFKOex3} cells, Supplementary Fig. 7). The 578^{VEGFKOex3} cells exhibited shorter perimeter morphology (Supplementary Fig. 8a-c) and reduced cell migration ability (supplementary Fig 8d). In addition, protein and mRNA expression of ARHGAP17 in 578^{VEGFKOex3} cells are upregulated compared to 578^{WT} cells (1.77-fold, Supplementary Fig.9a, b). These results are mostly identical to those of 231^{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* expression using

a publicly available dataset. The dataset of the METABRIC study³⁶ was downloaded from the cBioportal website³⁷, which consisted of clinical information, copy number alteration (CNA) and gene expression data of 1980 primary tumors of BC patients. Among the 1980 BC samples, 1468 had no CNA in *NRP1* and *ARHGAP17* gene 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* molecules³⁴; thus, distribution of *ARHGAP17* and *VEGFA/SEMA3A* ratio was shown in a scatter plot, as Supplementary Fig. 10a, and its correlation was estimated by Pearson's and Spearman's methods (Supplementary Table 6). The samples were stratified into 4 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. 5a, *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, Fig. 5b-g, Supplementary Table 7). 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 receptors⁵. 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 multi-function molecule that affects a variety of cells in the tumor microenvironment, not only in endothelial cells⁵. 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 ($231^{VEGFKOex3}$) cells (Fig. 1). Unlike bevacizumab-treated MDA-MB-231 cells, $231^{VEGFKOex3}$ 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-4³⁸, 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 cells³⁹. 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 cells⁴⁰. 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 outcome⁶. In our analysis using the METABRIC study data (Supplementary Fig. 10b-g), *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 (Supplementary Fig. 10b, 10f, Supplementary Table 7), 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. 5, *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 Supplementary Table 10, the association between HR status and bevacizumab treatment efficacy in clinical trials has been inconsistent⁴¹⁻⁴⁸. 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^{31,49-53}. 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 (Supplementary Fig. 11). 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)⁵⁴, the CpG island is mostly unmethylated in all BC cell lines. However, one probe at -290bp 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) (Supplementary Fig.12). 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^{55,56} 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 NRP1²³. As shown in the current study, long VEGF isoforms (VEGF165) have high affinity to NRP1 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

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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.

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Figure Legends

Figure 1

Knocking out of VEGF by CRISPR-Cas9 system.

(a) Cell proliferation assay using WST-8. Bevacizumab treatment did not produce any significant changes in proliferation of MDA-MB-231 cells. (b) Schematic diagram depicting VEGF isoforms and the locations of guide RNA (gRNA) on the *VEGF* locus. The gRNA was placed on a common exon of all VEGF isoforms. (c) Protein expression levels of VEGF in culture media of 231^{WT} cells and 231^{VEGFKO} cells. VEGF was not detectable in culture media of the VEGFKO cells. (d) Cell growth rate by direct counting. There was not any significant difference in the cell growth rate between 231^{WT} cells and 231^{VEGFKOex3} cells. The doubling times of the 231^{WT} and 231^{VEGFKOex3} cells in the exponential phase (Day2-4) were almost identical at 21.7 and 21.2 hours, respectively. In a, d and e, bars and error bars: mean ± standard deviation of triplicated experiments.

Figure 2.

VEGF knock out induced morphologic change in MDA-MB-231 cells.

Phase contrast microscope images of (a) wild type 231 cells (231^{WT}), (b) 231^{VEGFKOex3} cells, (c) bevacizumab-treated 231^{WT} cells, (d) rhVEGF165-treated 231^{VEGFKOex3} cells, (e) mock-vector-treated 231^{WT} cells, (f) 231^{SNRP} cells. (g) Western blotting shows NRP1 expression was knocked down by siRNA. (h) Comparison of perimeter of these cells measured using ImageJ software. *P*: *P*-values of the Mann-Whitney U test. (i) Cell migration assay

using Transwell chambers. Ten thousand cells were seeded into chambers. Twenty-four hours later, migrated cells in ten random microscopic fields were counted. Bars and Error bars: mean $\pm$ standard deviation of triplicated experiments. In **h-i**, *P*: p-values of Student's t-test, NS: not significant.

Figure 3.

Whole-genome expression analysis to identify VEGF/NRP1-dependent genes

To identify genes in the downstream of the VEGF/NRP1 signal, we conducted genome-wide gene expression profiling of 231^{WT} cells, 231^{VEGFKOex3} cells, and 231^{VEGFKOex3} cells exposed to rhVEGF165, using Agilent SurePrint G3 Human GE 8 $\times$ 60 K Microarray. Probe signal values of the genes were transformed into log2. **(a)** Scatter plot of 231^{WT} cells (x-axis) and 231^{VEGFKOex3} cells (y-axis). More than 2-fold up/down-regulated genes in 231^{VEGFKOex3} cells were shown as red dots (n=765). **(b)** Scatter plot of 765 up/down-regulated genes. X-axis: 231^{VEGFKOex3}/231^{WT} ratio in log2, Y-axis: (231^{VEGFKOex3}+rhVEGF165)/231^{WT} ratio in log2. Genes in which expression levels were restored 50 to 150% by rhVEGF165 treatment are shown as red dots (n=62). **(c)** Fold-change between 231^{VEGFKOex3} and 231^{WT} cells, and recovery by rhVEGF165 treatment for *ARHGAPs*, *ARHGEFs*, and *GDI*s. X-axis: fold change between 231^{VEGFKOex3} and 231^{WT} cells, Y-axis: fold change between 231^{VEGFKOex3} cells treated with rhVEGF165 and 231^{WT} cells. Blue dashed line: recovery rate equal to 0%. Dots in orange shade area: genes with more than 2-fold change and with recovery rate ranging from 50-150%.

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 $231^{VEGFKOex3}$ cells compared to 231^{WT} 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) 231^{WT} cells, (h) $231^{VEGFKOex3}$ cells, and (i) rhVEGF165-treated $231^{VEGFKOex3}$ cells, (j) rhVEGF121-treated $231^{VEGFKOex3}$ cells, (k) bevacizumab treated MDA-MB-231 cells, (n) 231^{SNRP1} cells and (m) control cells. In a, c, and e, bars and error bars: mean $\pm$ standard deviation of triplicated experiments. In a and c-e, *P*: *P*-values of Student's t-test, NS: not significant.

Figure 5.

Clinical significance of VEGF/NRP1/ARHGAP17 regulatory network in clinical tissue samples of HR(-) BC.

(a) Correlation between *VEGFA/SEMA3A* ratio and *ARHGAP17* expression in clinical tissue samples. Among 1980 samples of METABRIC study, 1486 without copy number alteration at *NRP1* nor *ARHGAP17* locus were selected for this analysis. The *VEGFA/SEMA3A* ratio and *ARHGAP17* expression was negatively correlated in pure HER2 and TN subtypes. Black and green dots: TN and pure Her2 subtypes by immunohistochemical four-gene subtyping, red dashed line and equation: regression line and its equation, Pearson's

correlation coefficient and its p-value are shown. Pearson's and Spearman's correlation coefficients, as well as the *P*-values for each subtype, are listed in Supplementary Table 6. Impacts of *VEGF* (**b**), *SEMA3A* (**c**), *NRP1* (**d**), *ARHGAP17* (**e**), *VEGF/SEMA3A* ratio (**f**), score of Cox proportional hazard model (**g**) on disease free survival of HR(-) BC are represented by Kaplan-Meier curves. *P*: *P*-value of Logrank test.

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 don't express VEGFR1/2, bevacizumab cannot block this VEGF-NRP1 interaction.

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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.

Competing financial interests

The authors declare that they have no competing financial interests.

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Figure 1

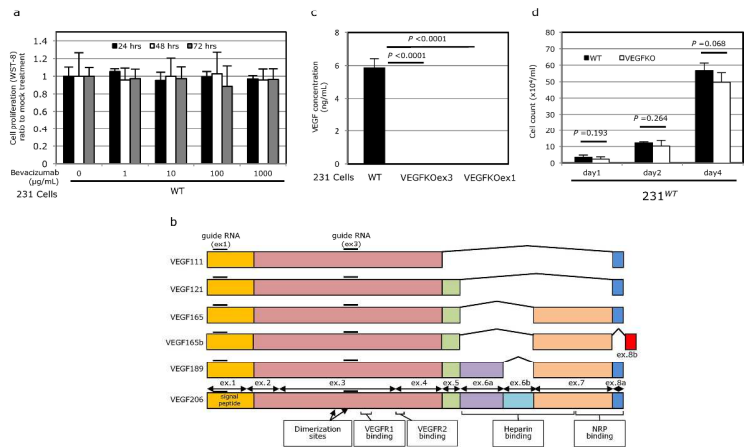


Figure 1

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Figure 2

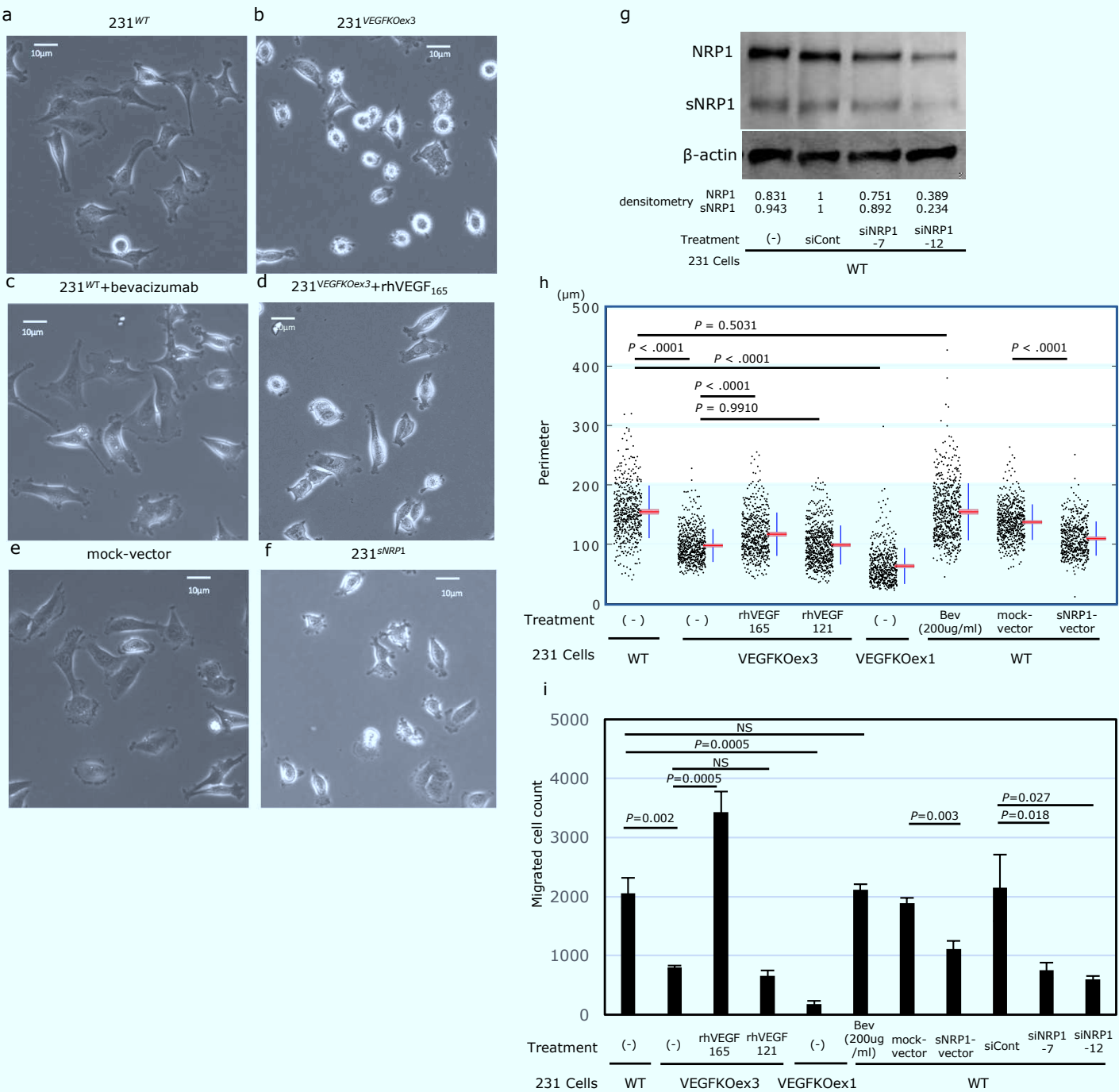


Figure 4

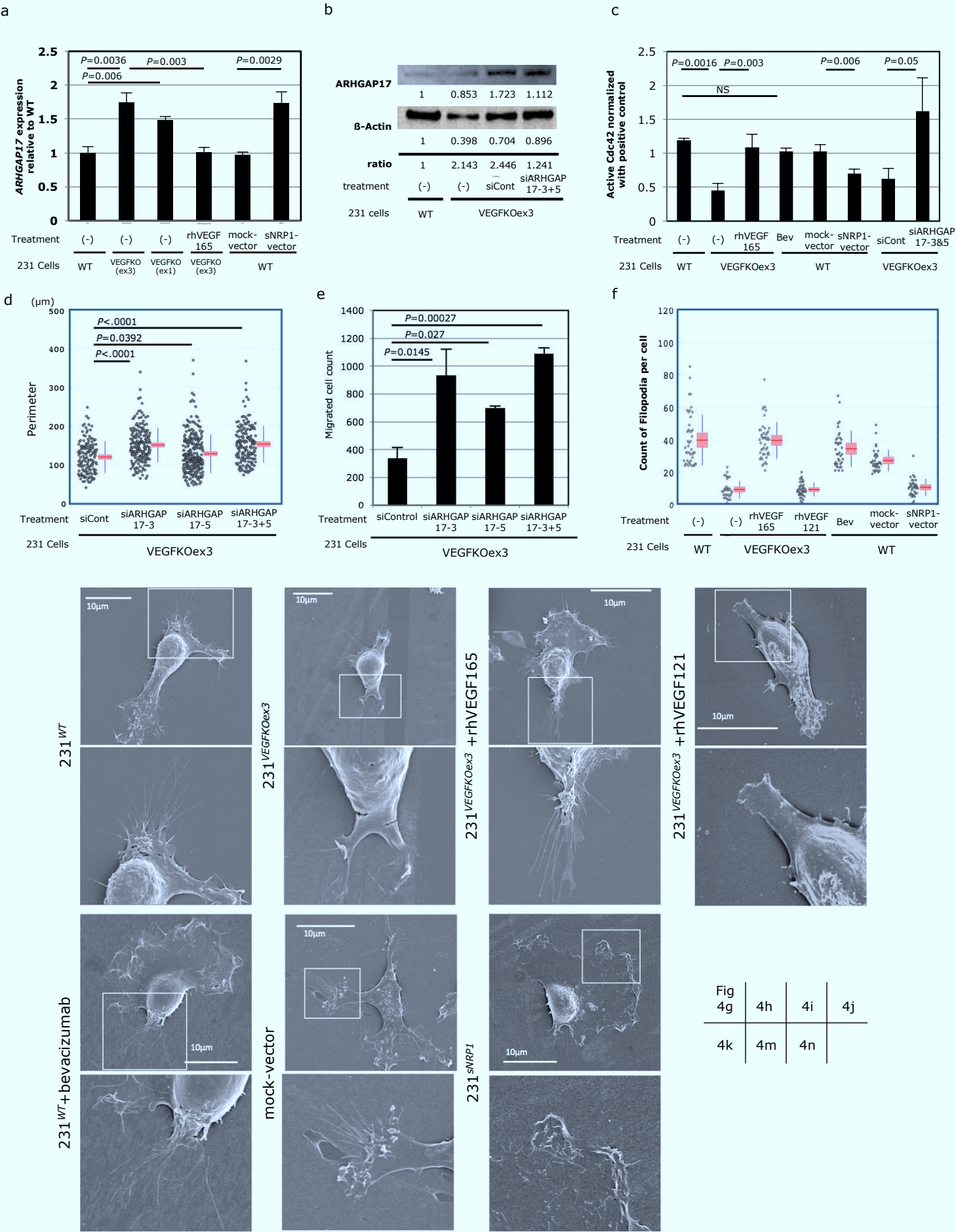


Figure 5

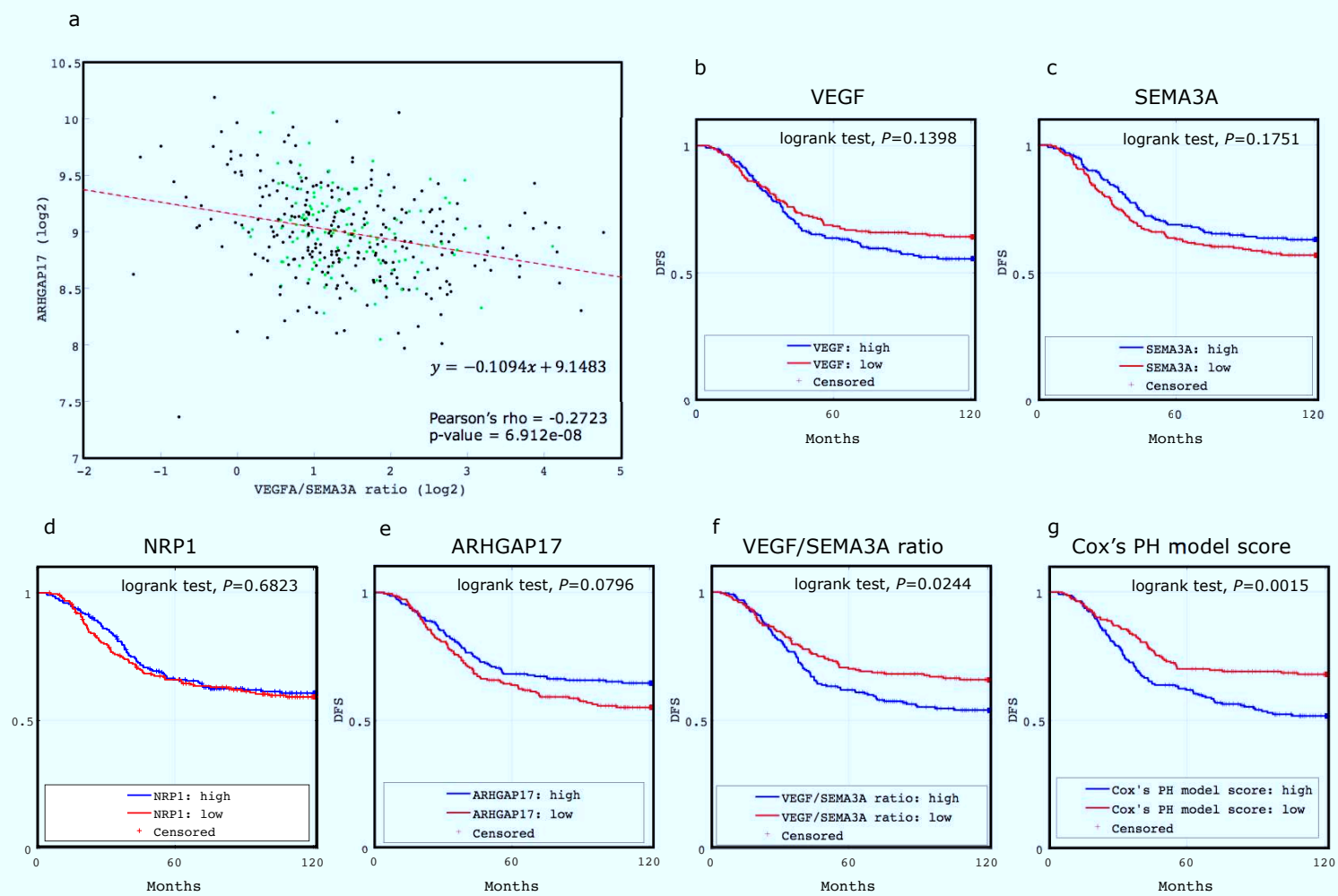


Figure 6

