Neuropilin-1-Mediated Vascular permeability factor / Vascular endothelial growth factor (VPF/VEGF)-Dependent Endothelial Cell Migration

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Running title: Neuropilin-1 in VPF/VEGF-mediated signaling

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Abbreviations:
VPF/VEGF, Vascular permeability factor / Vascular endothelial growth factor; EGF, epidermal growth factor; EC, endothelial cell; HUVEC, human umbilical vein endothelial cell; NRP-1, neuropilin-1; EGNP-1, the fusion protein with the extracellular domain of the EGF receptor and the transmembrane/intracellular domains of NRP-1; EGDR, the fusion protein with the extracellular domain of the EGF receptor and the transmembrane/intracellular domain of VEGFR-2; EGLT, the fusion protein with the extracellular domain of the EGF receptor and the transmembrane/intracellular domains of VEGFR-1; EGNP-1ΔSEA, the mutant of EGNP-1 by deleting the C-terminal three amino acids of NRP-1 (S-E-A-COOH); PI-3K, phosphatidylinositol 3-kinase; FACS, fluorescence-activated cell-sorting.
Neuropilin-1 (NRP-1) has been found to be expressed by endothelial cells and tumor cells as an isoform-specific receptor for VPF/VEGF. Previous studies were mainly focused on the extracellular domain of NRP-1 that can bind to VEGF$_{165}$ and thus enables NRP-1 to act as a co-receptor for VEGF$_{165}$, which enhances its binding to VEGFR-2 and its bioactivity. However, the exact functional roles and related signaling mechanisms of NRP-1 in angiogenesis are not well understood. In this study, we constructed a chimeric receptor, EGNP-1, by fusing the extracellular domain of EGF receptor to the transmembrane and intracellular domains of NRP-1, and transduced it into HUVEC with a retroviral expression vector. We observed that NRP-1/EGNP-1 mediates ligand-stimulated migration of HUVEC, but not proliferation. Our results show that NRP-1 alone can mediate HUVEC migration through its intracellular domain, and its C-terminal three amino acids (S-E-A-COOH) are essential for the process. We demonstrate that PI-3K inhibitor, Ly294002, and the p85 dominant negative mutant can block NRP-1-mediated HUVEC migration. NRP-1-mediated migration can be significantly reduced by overexpression of the dominant negative mutant of RhoA (RhoA-19N). In addition, Gq family proteins and G$_{b\gamma}$ subunits are also required for NRP-1-mediated HUVEC migration. These results show for the first time that NRP-1 can independently promote cell signaling in endothelial cells and also demonstrate the importance of last three amino-acids of NRP-1 for its function.

Angiogenesis, the formation of vascular networks by endothelial cells (ECs) sprouting from the vascular bed, occurs in many physiological or pathological processes (1). Vascular permeability factor / vascular endothelial growth factor (VPF/VEGF) plays a major role in the regulation of angiogenesis; it is regarded as a key contributor to the growth of cancer and vascular disease (2). VPF/VEGF activities are mediated by high-affinity receptor tyrosine kinases (RTKs) that are associated primarily with ECs (2). Two important VPF/VEGF binding RTKs, VEGFR-1 (Flt-1) and VEGFR-2 (KDR/Flk-1), have been identified, both of which are functionally active during angiogenesis. Recent studies have found a third VPF/VEGF receptor, neuropilin-1 (NRP-1), which is expressed by ECs and tumor cells (3, 4).

NRP-1 is a 130-135 kDa cell surface glycoprotein. It was originally characterized as a semaphorin III receptor that is important for guiding neural development (5, 6). There is also evidence that NRP-1 mediates angiogenesis. NRP-1-null mice were found to be embryonic lethal and exhibit
cardiovascular defect (7). Furthermore, overexpression of NRP-1 in mice resulted in excessive capillary and blood vessel formation and hemorrhaging in embryos (8). NRP-1 also contributes to tumor angiogenesis. Induction of NRP-1 expression in tumor cells in vivo resulted in larger and more vascular tumors (9). The stronger evidence for the role of NRP-1 in angiogenesis is its expression on ECs in the adult uterus (10) and on new vasculature in healing wounds (11).

Previously, it has been demonstrated that expression of NRP-1 on ECs enhanced the VEGF$_{165}$ binding to VEGFR-2 and VEGFR-2-mediated chemotactic activity of VEGF$_{165}$ (4). This suggests that NRP-1 is a co-receptor for VPF/VEGF, which has led to postulate that NRP-1 is involved in VPF/VEGF-mediated angiogenesis in vivo. Further studies on NRP-1 function have also provided evidence for its role in EC as a critical VEGFR-2 co-receptor that facilitates VPF/VEGF-mediated signaling through this tyrosine kinase-linked receptor (12). Additionally, the study in Dunning rat prostate carcinoma AT2.1 cells which express NRP-1 but not VEGFR-2 found that the expression of NRP-1 resulted in enlarged tumors associated with substantially enhanced tumor angiogenesis (9). A recent study showed that NRP-1 bound with high affinity to VEGFR-1 and that this interaction inhibited the binding of VEGF$_{165}$ to NRP-1 (13). Interestingly, a study by Bachelder et al. first demonstrated that NRP-1 supported VPF/VEGF autocrine function in cells lacking VEGFR-2 expression (14). All this evidence raises the possibility that NRP-1 functions, either alone or in concert with other tyrosine kinase-linked receptors, to transduce the VPF/VEGF signaling in different cells. However, neither of the above signaling pathways has been investigated to date.

NRP-1 contains a relatively large extracellular domain of 860 amino acids, a very short transmembrane domain of 23 amino acids and an intracellular domain of 40 amino acids. Previous studies were mainly focused on the extracellular domain that consists of five domains: a1/a2, b1/b2 and c (a MAM domain). The b1/b2 domains of NRP-1 contain the epitopes for both VPF/VEGF 165 and the positively charged C terminus of semaphorins (15, 16, 17) that are the structural basis for NRP-1 function as a VEGFR-2 co-receptor for VPF/VEGF signaling. Research from the nervous system showed that the intracellular domain of NRP-1 was not required for semaphorin signaling (16), and it did not contain sequences predictive of enzymatic activity nor sequences predicted to be involved in coupling to intracellular signaling molecules. Nevertheless, the transmembrane and intracellular domains of NRP-1 share > 90% amino acid identity across species (18, 19), which suggests an important role for this domain in terms of the NRP-1 functions. Moreover, the C-terminal three amino acids of NRP-1 (S-E-A-COOH), which are conserved from Xenopus to human, were responsible for binding to a PSD-95-Dlg/ZO (PDZ) domain (20, 21).
Because VEGFR-1, VEGFR-2, and NRP-1 are all expressed on ECs, it is difficult to delineate the distinct biological functions and signaling pathways of each induced in ECs by VPF/VEGF. Therefore, to elucidate the respective roles of these receptors in ECs, an approach engineering the chimeric construct of each of the receptors by replacing the extracellular domain of each with the extracellular domain of epidermal growth factor receptor (EGFR), was established previously in our laboratory (22). In this study, using a chimeric receptor EGNP-1 (fusing the extracellular domain of the EGFR and the transmembrane/intracellular domains of NRP-1), we examined the distinct biological function of the intracellular domain of NRP-1 and its signaling pathways in vascular endothelium. We found that NRP-1 alone can mediate HUVECs migration but not proliferation, and the C-terminal three amino acids of NRP-1 (S-E-A-COOH) are required for NRP-1-mediated HUVEC migration. The activation of RhoA can be mediated by NRP-1 through Gq family proteins, Gβγ subunits and PI-3K pathways that are required for HUVEC migration.

EXPERIMENTAL PROCEDURES

Materials – Human EGF (h-EGF), human VEGF (h-VEGF), primary HUVECs, EGM-MV bullet Kit and EC Basic Medium (EBM) were purchased from Clonetics (San Diego, CA). Mouse monoclonal antibody against the EGFR N-terminal domain, goat polyclonal antibody against the NRP-1-C-terminal domain and rabbit polyclonal antibody against RhoA were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). [3H]Thymidine was purchased from PerkinElmer Life Sciences (Boston, MA). Transwell plate insert was purchased from Fisher Scientific (Pittsburgh, PA). Calcein-AM was purchased from Molecular Probes (Eugene, OR). Pertussis toxin and Ly294002 were purchased from Calbiochem.

Cell culture – HUVECs were cultured as described previously (22). HUVECs were grown on 30 μg/ml vitrogen-coated dishes in EGM-MV Bullet Kit (5% fetal bovine serum [FBS] in EBM with 12 μg/ml bovine brain extract, 1 μg/ml hydrocortisone, and 1 μg/ml GA-1000). HUVECs (passage 3 or 4) that were ~80% confluent were used for most experiments. Cells were serum-starved in 0.1% FBS in EBM for 24 h before testing.
Construction and overexpression of chimeric fusion receptor EGNP-1 – Chimeric receptor EGNP-1 and its mutant EGNP-1ΔSEA were constructed by fusing the extracellular domain of EGFR (N-terminus) with the transmembrane and intracellular domains of NRP-1 (C-terminus) and NRP-1ΔSEA (C-terminus) respectively. PCR were carried out to generate the required fragments. For preparing the N-terminal portion of the EGF receptor, template EGDR (a chimeric receptor that consists of the extracellular domain of EGFR and the transmembrane and intracellular domains of KDR was constructed previously in our laboratory [22]) was cut from pMMP-EGDR with NcoI and NotI. 5’ primer (CCA TGG GTC GAC CAGC ATG GGA CCC TCC GGG) contains NcoI enzyme restriction sequence just before the translation start site (ATG). A Apal enzyme restriction sequence was inserted in the 3’ primer (GGG CCC ATT CGT TCC TGT CGG AAG TTC) of EGFR, the nucleotide sequence ending at amino acid 640 was fused to a sequence of NRP-1 beginning at amino acid 856. NRP-1 was used as a template to get the C-terminal portion of NRP-1 and NRP-1ΔSEA respectively. The 5’ primer (GGG CCC ATC CTC ATC ACC ATC ATA GCC) began at amino acid 856 of NRP-1 with an insertion of Apal enzyme restriction sequence. In the 3’ primer (GCG GCC GC TCA TGC CTC CGA ATA AGT ACT CTG) of NRP-1, a Notl site was created immediately after the stop code (TGA). As for the C-terminal portion of NRP-1ΔSEA, the 3’ mutagenesis primer was GCG GCC GCT CAA TAA GTA CTC TGT GTA TTC AGT TTG TC, in which a stop code (TGA) was inserted before the C-terminal three amino acids of NRP-1 (S-E-A-COOH) and a NotI site was created immediately after the stop code. All the PCR products were confirmed by DNA sequence analysis. These fragments were then ligated respectively to obtain EGNP-1 (EGFR/NRP-1) and EGNP-1ΔSEA (EGFR/NRP-1ΔSEA) and further subcloned into retroviral vector pMMP to yield pMMP-EGNP-1 and pMMP-EGNP-1ΔSEA.

For preparing retrovirus, 293T cells were seeded at 3 x 10^6 cells per 100-mm plate. After 24 h, DNA transfection was performed with the Effectene™ transfection reagent (QIAGEN, Valencia, CA), and with 2 μg of targeted gene (pMMP-EGNP-1, pMMP-EGNP-1ΔSEA, pMMP-LacZ, etc.), 1.5 μg of pMD.MLV gag.pol and 0.5 μg of pMD.G (encoding the cDNAs of the proteins that are required for virus packing). The medium was changed after 16 h. The retrovirus was collected 48 h after transfection and used immediately for infection or frozen at -70°C. For transducing the target gene into HUVECs, the cells were seeded at a density of 2 x 10^5 per 100-mm plate. Infection was carried out by adding 2.5 ml of retrovirus solution and 7.5 ml of fresh medium (~2 x 10^7 plaque-forming units/ml), together with 10 μg/ml Polybrene to cells. The medium was changed after 16 h and the cells were ready for experiment 48 h after infection.
Immunoprecipitation and Western Blotting – 48 h after infection, HUVECs were lysed with cold immunoprecipitation buffer (50 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 1% NP-40, 0.5% Sodium deoxycholate, 0.1% SDS), which contains 1 mM PMSF, 1 µg/ml leupeptin, 0.5% aprotinin, and 2 µg/ml pepstatin A. 500 µg of lysate protein was incubated with 1 µg of antibody at 4°C for 2 h, and then with 50 µl of protein G-conjugated agarose-beads at 4°C for 2 – 5 h. After washing the beads with the same buffer, immunoprecipitates were resuspended in 2 x SDS sample buffer for Western blot analysis. All experiments were repeated at least three times.

FACS analysis – For detaching cells, serum-starved HUVECs were incubated with 4 ml of collagenase solution (0.2 mg/ml collagenase, 0.2 mg/ml soybean trypsin inhibitor, 1 mg/ml BSA, and 2 mM EDTA in PBS) at 37°C for 30 min. Cell pellets were washed with cold PBS containing 0.1% BSA and 5 x 10⁵ cells were suspended in 40 µl of the same buffer containing 4 µg of mouse anti-EGFR-N antibody or mouse IgG and incubated on ice for 1 h. The cells were washed again and resuspended in 40 µl of the same buffer with 2.5 µg/ml fluorescein isothiocyanate - conjugated anti-mouse IgG antibody. After incubation at 4°C for 30 min, cells were washed and resuspended in 400 µl of the same buffer. FACS analysis was carried out in a Calibur instrument (BD Biosciences) with Cellquest software.

Migration assay - Serum-starved HUVECs (with or without retrovirus infection) were stained with Calcein-AM (25 µg Calcein-AM was dissolved in 5 µl DMSO and then was added into 4ml EBM containing 0.1% BSA per 100 mm plate) at 37°C for 30 min. The cells were then detached from tissue culture plates as described in FACS analysis. Then, cells were seeded as 1 x 10⁵ per well in 500 µl EBM with 0.1% FBS into the transwells coated with vitrogen (30 µg/ml), and the transwells were inserted into a 24-well plate containing 750 µl of the same medium. The cells were incubated at 37°C for 45 min to allow them to attach. Afterwards, VPF/VEGF or EGF was added at a final concentration of 10 ng/ml and an additional 2 h incubation was performed. The migrated, stained cells were counted in a spectrofluorometer (Spectrafluor; TECAN) with Delta Soft 3 software. The standard curve was made in the same condition with cells over a range of 3 X 10³ to 1 X 10⁵ cells/well. For migration inhibition experiments, various inhibitors were added at different times before VPF/VEGF or EGF stimulation as indicated. Data are expressed as the mean ± SD of quadruplicate values. All experiments were repeated at least three times.
**Proliferation assays** – Proliferation assay was carried out as described previously (22). 2 x 10^3 cells were seeded into each well in a 24-well plate. After 24 h, the cells were infected with 200 µl of retrovirus in 800 µl fresh medium as described above. After 2 days, cells were Serum-starved for 24 h and then stimulated with 10 ng/ml VPF/VEGF or 10 ng/ml EGF for 20 h. The assay was carried out as previously described (22). 1 µCi/ml of [³H]Thymidine was added to each well. After 4 h incubation at 37°C, cells were washed with cold PBS, fixed with 100% cold methanol at 4°C for 15 min, precipitated with 10% cold trichloroacetic acid (TCA) at 4°C for 15 min, washed with ddH₂O three times, and lysed with 200 µl of 0.1 N NaOH at room temperature for 30 min. [³H]Thymidine incorporation was measured in scintillation solution. Data are expressed as the mean ± SD of quadruplicate values. All experiments were repeated at least three times.

**RhoA activation assay** – RhoA activation assay was carried out as described previously (23). The glutathione-S-transferase (GST)-Rhotekin Rho binding domain (TRBD) fusion protein was kindly provided by Dr. Martin Schwartz (Scripps Institute). In brief, pGST-TRBD bacteria were grown and induced with isopropylthiogalactoside. The bacterial suspensions were divided by 50 ml of aliquot and then harvested and frozen at -80°C. To prepare the GST-TRBD beads, each aliquot of frozen bacteria was resuspended in 2 ml of cold PBS, and then 20 µl of 1 M dithiothreitol (DTT), 20 µl of 0.2 M PMSF, and 40 µl of 50 mg/ml lysozyme were added and incubated on ice for 30 min. Afterwards, 225 µl of 10% Triton X-100, 22.5 µl of 1M MgCl₂, 22.5 µl of 2000 KU/ml DNAse were added and the sample was incubated on ice for another 30 min. The supernatant was collected and incubated with 100 µl glutathione-coupled Sepharose 4B beads (Pharmacia Biotech) at 4°C for 45 min. The beads were then washed with bead washing buffer (PBS with 10 mM DTT and 1% Triton X-100) and resuspended in the same buffer to emit 50% bead slur.

Meanwhile, 24 h SEAum-starved HUVECs with retroviral infection were stimulated with 10 ng/ml of EGF at different times. Stimulation was stopped by adding cold PBS. Then the cells were lysed with lysis buffer (150 mM NaCl, 0.8 mM MgCl₂, 5 mM EGTA, 1% IGEPAL, 50 mM HEPES, pH 7.5, 1 mM PMSF, 10 µg/ml leupeptin, and 10 µg/ml aprotinin). The supernatant was isolated and incubated with 50 µl of GST-TRBD beads at 4°C for 45 min. Protein bound to beads was washed with AP wash buffer (50 mM Tris-HCl, pH 7.2, 1% Triton X-100, 150 mM NaCl, 10 mM MgCl₂, 1 mM PMSF, 10 µg/ml leupeptin, and 10 µg/ml aprotinin) and analyzed by SDS-PAGE with antibody.
against RhoA. For inhibition experiments, inhibitors were added respectively as indicated. All experiments were repeated at least three times.

RESULTS

Construction, transduction, and recombinant expression of EGNP-1 chimeric receptor – Previously, our laboratory developed EGLT and EGDR fusions with the extracellular domain of EGFR to the transmembrane/intracellular domains of VEGFR-1 and VEGFR-2 respectively and expressed them in HUVEC with a retrovirus expression vector (22-24). At 80% confluence, HUVECs do not express EGFR, and therefore, the fusion receptor can be used to distinguish the signaling pathway that is mediated by VEGFR-1 and VEGFR-2. In this study, to investigate the biological activities and the specific signaling events mediated by NRP-1 in EC, we developed a chimeric receptor, EGNP-1, by fusing the extracellular domain of EGFR to the transmembrane and intracellular domains of NRP-1 (Fig. 1a). Using a retroviral expression vector pMMP, the chimeric receptor EGNP-1 was transduced into HUVEC. Cells transduced with LacZ were used as control. Western blot (IB) analysis was performed on these cells lysate with an antibody to the C-terminus of NRP-1 (anti-NRP-1-C). The results showed the specific bands of endogenous NRP-1 in both cells, but the EGNP-1 band only in EGNP-1 transduced HUVECs (Fig. 1b, the left two lanes). Immunoprecipitation (IP) with an antibody against EGFR N-terminal (anti-EGFR-N) and then Western Blot with anti-NRP-1-C were also carried out and further demonstrated that the clear specific band of EGNP-1 that expressed only in EGNP-1 transduced HUVECs (Fig. 1b, the two right lanes). Of importance, the expression of endogenous NRP-1 and recombinant EGNP-1 expression are almost in similar levels.

To determine the expression of EGNP-1 on HUVEC surfaces, FACS analysis was performed using a mouse monoclonal antibody specific for the N-terminus of EGFR and normal mouse IgG as control. HUVECs were transduced with LacZ (negative control), EGDR (positive control), and EGNP-1 respectively. The results showed that about 90% of transduced cells expressed EGDR, and more than 70% of transduced cells expressed EGNP-1 (Fig. 1c). EGFR expression was not detected in LacZ transduced HUVECs as previously shown (22).

EGNP-1 mediates HUVEC migration, but not proliferation – First, we examined whether EGNP-1 could mediate the migration and proliferation of HUVEC. Migration assays were carried out on HUVECs transduced with EGNP-1, EGDR, EGLT and LacZ respectively. As shown in Fig. 2a I,
EGF induced the migration to an almost equivalent level in HUVECs that had been transduced with either EGNP-1 or EGDR, but not to that of native HUVECs and HUVECs transduced with EGLT or LacZ. All of the cells showed a similar response to VPF/VEGF stimulation, indicating that the cells had good responsiveness. The results from Western Blot analysis demonstrated all the chimeric receptors were expressed at relatively comparable levels in HUVECs (Fig. 2a III). These results indicated that EGNP-1 can mediate the ligand-dependent migration of HUVEC. Furthermore, proliferation assays were also performed on these HUVECs as above. As shown in Fig. 2a II, VPF/VEGF treatment stimulated more than 3-fold in parental HUVECs and EGF treatment promotes approximately 4 fold in EGDR transduced HUVECs in trichloroacetic acid-precipitable [³H]Thymidine incorporation. On the other hand, there was no response in EGF stimulated EGNP-1 or LacZ transduced HUVECs, or parental HUVECs. The results suggest that EGNP-1 is not required for ligand-dependent HUVEC proliferation.

To quantitatively characterize the medication function of EGNP-1 in HUVEC migration, we infected HUVECs by varying the number of retrovirus particles containing EGNP-1. Fig. 2b II illustrated that, the transduced cells expressed progressively increased levels of EGNP-1. Stimulating the transduced cells with EGF, indicated that there was a positive correlation between its migration and levels of EGNP-1 (Fig. 2b I, Fig. 2b II). This quantitative result further demonstrated that NR1/EGNP-1 mediates EGF-induced HUVEC migration.

In order to examine whether EGNP-1 alone can mediate migration of HUVEC, EGDR mutants, EGDR (Y951F) and EGDR (Y1059F) (site-directed mutagenesis in tyrosine residue 951 and 1059 of EGDR essential for VPF/VEGF induced HUVEC migration and proliferation, respectively (24)), and EGLT mutants, EGLT (793stop) (a stop codon at tyrosine residue 794 of EGLT essential for EGLT’s antiproliferation effect (22)) and EGLT (824stop) (a truncation mutant immediately before the VEGFR-1 kinase domain) were respectively transduced into HUVECs with and without EGNP-1. We found that the migration of HUVECs co-transduced with EGNP-1 and EGDR mutants or EGNP-1 and EGLT mutants showed no difference from that of cells transduced with EGNP-1 alone (Fig. 3a I). In all the cases, the expression of EGNP-1 was at similar levels (Fig. 3a II). To confirm these results, we inhibited the VEGFR-2 function by utilizing anti-VEGFR-2 antibody on parental or EGNP-1 transduced HUVEC, respectively. The results indicated that VEGFR-2 antibody inhibited native HUVECs migration induced by VPF/VEGF but not EGNP-1-transduced HUVECs migration induced by EGF (Fig. 3b). Taken together, these data demonstrate that the function of NR1/EGNP-1-mediated HUVEC migration can be VEGFR-2 or VEGFR-1 independent.
**Signaling downstream of NRP-1–mediated endothelial cell migration** - To examine the role of PI-3K signaling pathways in NRP-1-mediated HUVEC migration, Ly294002, a PI-3K inhibitor, was used. After treatment of EGNP-1 transduced HUVECs with Ly294002, a significant decrease of HUVEC migration in response to EGF was observed (Fig. 4a). PI-3K contains a kinase subunit (p110) and an inhibitory subunit (p85) and functions in tyrosine kinase receptor signaling pathways (25). Ligand-activated receptors interact with the p85 subunit, releasing the p110 subunit in an active form. We used the dominant negative mutants of the p85 subunit (p85 DN) and the constitutive activated mutant of PI-3K (p110CAAX) to determine whether PI-3K is required for EGNP-1-mediated migration. The results showed that the co-transduced HUVECs expressed similar levels of EGNP-1 (Fig. 4b II), but the co-transduction of HUVECs with EGNP-1 and p85(DN) reduced EGF-stimulated migration as compared to that of HUVECs co-transduced with EGNP-1 and LacZ (Fig. 4b I). However, the p110CAAX increased EGNP-1-mediated migration induced by EGF. These results indicated that EGNP-1-mediated ligand-dependent migration of HUVEC is through the PI-3K signaling pathway.

Previous work on the nervous system has shown that NRP-1 may be involved in the regulation of vesicular trafficking by association with the G-protein-coupled signaling pathways (20). Hence, we analyzed whether G proteins are involved in the signaling of NRP-1/EGNP-1-mediated HUVEC migration. It has been shown that pertussis toxin inhibits VEGFR-1-mediated microphage migration and HUVEC anti-proliferation stimulated by VPF/VEGF (26, 27), which suggest that pertussis toxin-sensitive G-proteins may participate in VPF/VEGF-dependent signaling. Recent studies from our laboratory have shown that Gq family proteins are important for VPF/VEGF-stimulated HUVEC proliferation and migration (28). Serum-starved EGNP-1-transduced HUVECs were pretreated with 100 ng/ml of pertussis toxin for 16h or 1 µM TATFGp (a cell permeable fusion peptide TAT-FLAG-Gp, which consists of TAT peptide, an epitopeFLAG sequence and Gp Antagonist-2A. Gp Antagonist-2A can specifically inhibit function of Gq family proteins *in vitro* (28, 29, 30)) for 5 min respectively and then stimulated with EGF. The data show that pretreatment with pertussis toxin did not have any effect on EGNP-1-mediated HUVEC migration, but TATFGp significantly decreased the migration (Fig. 5a). These results suggest that Gq family proteins are involved in EGNP-1-mediated HUVEC migration. It is known that after activation, heterotrimeric G proteins dissociate into α and βγ subunits that can trigger several downstream signaling pathways (31, 32). Gβγ is also known to be involved in VEGFR-1 and VEGFR-2 mediated signaling pathways (23, 27). Therefore, we further tested the role of free Gβγ subunits in NRP-1/EGNP-1-mediated HUVEC migration. hβARK1(495)
corresponds to the C-terminal domain of human βARK1 that physically interacts with free Gβγ and therefore acts as a specific intracellular Gβγ antagonist by inhibiting Gβγ-mediated downstream events (33, 34). Our results showed that overexpression of hβARK1(495) almost completely reduced the migration of HUVEC mediated by EGNP-1 (Fig. 5b I) although approximately the same levels of EGNP-1 were expressed in all the co-transduced cells (Fig. 5b II), indicating that Gβγ subunits are required for EGNP-1-mediated HUVEC migration.

The RhoA family of the small GTPase superfamily has been shown to play an important role in cell growth and migration (35). Recently, we have reported that CDC42 and Rac1 are required for VEGFR-1-mediated HUVEC anti-proliferation (27), whereas RhoA and Rac1 are important for VEGFR-2-mediated HUVEC migration (23). With the dominant negative mutants of CDC42 (CDC42-17N), Rac1 (Rac1-17N), and RhoA (RhoA-19N), the fragments encoding the genes were previously subcloned to a retroviral vector pMMP respectively in our laboratory (27), we examined whether Rho family proteins were involved in NRP-1 mediated HUVEC migration. HUVECs were co-transduced with EGNP-1 and CDC42-17N, Rac1-17N, or RhoA-19N respectively. Fig. 6 II showed all the co-transduced cells expressed EGNP-1 at relatively comparable levels. Fig. 6 I shows that compared to LacZ transduced cells, the CDC42-17N or Rac1-17N transduced cells did not influence EGNP-1-mediated HUVEC migration, but the RhoA-19N transduced HUVEC showed significant inhibition of ligand-induced migration. Taken together, our results suggest that RhoA is involved in ligand-dependent migration of HUVEC mediated by EGNP-1.

**NRP-1/EGNP-1 and related intracellular signaling molecules mediate activation of RhoA** – As the dominant negative mutant of RhoA (RhoA-19N) decreases EGF-induced HUVEC migration mediated by EGNP-1, we further examined whether EGNP-1 can mediate ligand-dependent RhoA activation. The activity of RhoA was measured by a pull-down assay using a GST-TRBD fusion protein that binds only to the GTP-bound form of RhoA. Serum starved HUVECs transduced with EGNP-1 or LacZ respectively were stimulated with 10ng/ml EGF for different intervals as indicated. Cellular extracts were incubated with freshly prepared GST-TRBD beads. Proteins bound to the beads were subjected to Western Blot analysis using the antibodies against RhoA. The results show that RhoA was not activated by EGF in LacZ transduced HUVECs, but was activated by EGF in EGNP-1 transduced HUVECs as early as 0.5 min and remains high at 5 min after EGF stimulation (Fig. 7a).

To test the signaling molecules that mediate RhoA activation, EGNP-1 transduced HUVECs were pretreated with a PI-3K inhibitor, Ly294002, for 5 min and then stimulated with 10 ng/ml EGF
for 1 min. Cellular extracts were used to measure the activation of RhoA. The data show that Ly294002 inhibited RhoA activation in EGNP-1 transduced HUVECs after EGF stimulation (Fig. 7b I). Furthermore, the dominant negative mutant of PI-3K, p85DN completely inhibited EGF-stimulated EGNP-1-mediated RhoA activation (Fig. 7b II). These results indicate that PI-3K is a key mediator of EGNP-1-mediated RhoA activation.

As described earlier, Gq family proteins and Gβγ subunits are necessary for NRP-1/EGNP-1-mediated HUVEC migration, therefore, we examined whether Gq family proteins have any effect on RhoA activation mediated by EGNP-1. Serum-starved HUVECs transduced with EGNP-1 were pretreated with pertussis toxin for 16h or TATFGp for 5 min followed by stimulation with EGF for 1 min. Cellular extracts were subjected to RhoA activation assay. The results indicated that TATFGp inhibited the RhoA activation mediated by EGNP-1 but pertussis toxin did not (Fig. 7c I). Subsequently, we examined whether Gβγ subunits are required for RhoA activation. Serum-starved HUVECs co-transduced with EGNP-1 and LacZ or hβARK1(495) were stimulated with 10 ng/ml EGF. Cellular extracts were then subjected to the RhoA activation assay. The data illustrated that overexpression of hβARK1(495) inhibited the activation of RhoA (Fig. 7c II), thus indicating that Gβγ subunits are required for EGNP-1-mediated RhoA activation.

The C-terminal three amino acids of NRP-1 (S-E-A-COOH) are essential for ligand-induced EGNP-1-mediated HUVEC migration – To confirm the specific signaling transduction induced by NRP-1 in HUVEC, a chimeric mutant receptor was created by deleting the C-terminal three amino acids of NRP-1 (S-E-A-COOH) and was designated EGNP-1ΔSEA (Fig. 1a). HUVECs transduced with LacZ, EGNP-1, and EGNP-1ΔSEA expressed the equal amounts of NRP-1, and accordingly expressed the equal amounts of EGNP-1 and EGNP-1ΔSEA (Fig. 8a I). In order to confirm that the mutant protein is expressed on the cell surface as a receptor, the transduced cells were subjected to FACS analysis with anti-EGFR-N and normal mouse IgG as a control. The result indicated that about 70% of HUVECs infected with EGNP-1ΔSEA and about 90% of HUVECs infected with EGDR expressed the receptors on the cell surface, whereas no EGFR was detected on HUVECs transduced with LacZ (Fig. 8a II).

Next, we investigated the effect of the mutation on EGNP-1-mediated HUVEC migration. As shown in Fig. 8b I, in HUVECs transduced with EGNP-1, EGF stimulation increased migration by more than 2 folds, whereas, HUVECs transduced with EGNP-1ΔSEA or LacZ did not respond to EGF. Fig. 8b II showed that the transduced cells expressed similar levels of EGNP-1 or EGNP-1ΔSEA.
However, HUVECs transduced with EGNP-1\(\Delta\)SEA showed a similar response to VPF/VEGF stimulation, indicating that the lack of migration response of EGNP-1\(\Delta\)SEA is not due to the defect of the cell. These results indicated that the C-terminal three amino acids of NRP-1 (S-E-A-COOH) are essential for ligand-induced HUVEC migration.

We further examined whether EGNP-1\(\Delta\)SEA has any effect on NRP-1-mediated RhoA activation in HUVEC. RhoA activity assay was carried out in HUVECs transduced with EGNP-1\(\Delta\)SEA. As shown in Fig. 8c, EGNP-1\(\Delta\)SEA did not mediate RhoA activation in response to EGF stimulation. This suggested that the C-terminal three amino acids of NRP-1 (S-E-A-COOH) are required for the signaling transduction of EGNP-1-mediated HUVEC migration.

DISCUSSION

Research on neuropilins and their biological functions is just at the beginning. Although it is known that NRP-1 acts as an important modulator of VPF/VEGF function during vasculogenesis and angiogenesis, its exact functional roles and related signaling mechanisms in these processes are still unclear (4,9,12,14). This study has elucidated some of the functional roles of NRP-1 in EC migration and explored related signaling mechanisms, the implications of which are discussed below.

This study provides direct evidence for the mediation function of NRP-1 in VPF/VEGF-dependent migration in HUVECs. To probe the functional role of NRP-1 in early-passaged ECs, we used an approach previously established in our laboratory (22) to develop a chimeric receptor EGNP-1 by fusing the extracellular domain of the EGF receptor with the transmembrane and intracellular domains of NRP-1. In this study, we showed for the first time that EGNP-1 mediates EGF-induced HUVEC migration but not proliferation. Furthermore, we identified that EGNP-1-mediated HUVEC migration is dose-dependent. This phenomenon, although observed by using different experimental approaches and different types of endothelium, agrees with and extends a recent study by Matthias et al. (11) where it was described that the migration of human microvascular endothelial cells to VEGF\(_{165}\) was severely inhibited \textit{in vitro} in the presence of anti-NRP-1 antibody and this inhibition was dose-dependent. These converging findings consistently support that NRP-1 mediates VPF/VEGF-induced EC migration.

This study examined whether NRP-1 alone can mediate EC migration. There are three lines of evidence in our results that support this functional role of NRP-1. First, we found that EGF stimulates a nearly equivalent extent increase of migration in HUVECs transduced with EGNP-1 or EGDR
(another chimeric receptor identified to represent the function of VEGFR-2 under EGF stimulation (22)). Secondly, there was no effect on EGF-stimulated HUVEC migration when HUVECs were cotransduced with EGNP-1 and EGDR mutants or EGLT mutants. EGDR(Y951F) is a mutant of EGDR in tyrosine residue 951 of VEGFR-2 that completely abolishes the EGF-(up to 100 ng/ml) induced migratory activity in HUVECs transduced with EGDR(Y951F) (Fig. 3a) (24). Finally, blocking VEGFR-2 with an anti-VEGFR-2 antibody on EGNP-1-transduced HUVECs had no effect on EGF-induced HUVEC migration. Overall, these results indicate that NRP-1/EGNP-1 can mediate ligand-dependent HUVEC migration as an independent receptor. This finding is supported by a number of previous studies. Neuropilin binding with VPF/VEGF isoform enhanced breast carcinoma cell (lacking the expression of VEGFR-2) survival by stimulating the PI-3K pathway (14). However, the present study seems to contradict our previous result (4) that showed no migration response to VEGF165 when NRP-1 alone was expressed on porcine aortic endothelia (PAE) cells. The possible explanation is that PAE cells do not normally express detectable levels of VEGFR-2, VEGFR-1 or NRP-1 and do not respond to VPF/VEGF (4, 36). Therefore, PAE cells may lack related signaling molecules with the absence of a receptor and may be less representative of vascular endothelium than early-passaged HUVECs used in this study. On the other hand, VEGFR-2 bound to VEGF165 more efficiently in cells expressing NRP-1, and this potentiating effect was subsequently translated into a better migratory response to VEGF165 as compared to the migratory response of cells expressing VEGFR-2 but not NRP-1. Thus, NRP-1 seems to function as an enhancer of VEGFR-2 activity in the presence of VEGF165. This effect is probably due to a complex formation between VEGFR-2 and NRP-1 (12).

This study is the first report that demonstrates the C-terminal three amino acids of NRP-1 (S-E-A-COOH) are essential for NRP-1-mediated HUVEC migration. It is known that the C-terminal three amino acids of NRP-1 (S-E-A-COOH) are responsible for interaction with the PDZ domain-containing C-terminal two-thirds of NRP-1-interacting protein (NIP) (20, 21). One of the important roles of PDZ domain-containing proteins is to act as molecular adapters that target proteins to proper subcellular compartments or assemble signal transduction components into closely associated protein complexes (37,38,39). The physical interaction and co-localization of NRP-1 and NIP in the nervous system suggested that NRP-1 functionally interacts with NIP (20). Recently, NIP has been independently cloned as RGS-GAIP-interacting protein (GIPC), where it was identified by virtue of its interaction with the C terminus of RGS-GAIP (a Gαi3-associated protein located to the membrane of clathrin-coated vesicles) and was suggested to participate in the regulation of clathrin-coated vesicular
trafficking by association with the G-protein-coupled signaling complex (21). In view of these findings as well as those of ours, we postulate that there may be a PDZ domain-containing NIP in HUVEC that participates in the regulation of NRP-1-mediated migration signaling by interacting with the C-terminal three amino acids of NRP-1 (S-E-A-COOH).

The involved signaling molecules for NRP-1/EGNP-1-mediated HUVEC migration were investigated in this study. We found that PI-3K inhibitor Ly294002 and the dominant negative mutant of p85 (p85DN) inhibit EGNP-1-mediated HUVEC migration induced by EGF, but the constitutive activated mutant of PI-3K (p110CAAX) expression results in more migration. These observations indicate that PI-3K is involved in the NRP-1-mediated migration signaling pathway, which is consistent with the findings by Bachelder et al. that neuropilin activates the PI-3K pathway induced by VPF/VEGF in metastatic breast carcinoma cells that is important for the survival of these cells (14). The experiments in G-proteins showed that Gp Antagonist-2A, a pharmacological antagonist of Gq proteins, causes almost complete inhibition of EGNP-1-mediated HUVEC migration induced by EGF, and further showed that Gβγ-sequestering peptide hβARK(495) significantly inhibited EGNP-1-mediated HUVEC migration. These data demonstrate that Gq family proteins are involved in VPF/VEGF-stimulated NRP-1-mediated HUVEC migration and Gβγ subunits are required for this signaling pathway. In corroboration with the previous study in our laboratory that heterotrimeric G protein Gq/11 and Gβγ subunits are required for VEGFR-2-mediated EC migration (23), this study further confirms that Gq family proteins and Gβγ subunits are required for VPF/VEGF-stimulated EC migration. The current study found that the RhoA dominant negative mutant, RhoA-19N, significantly inhibits EGF-stimulated EGNP-1-mediated HUVEC migration. This indicates that the small GTPase RhoA is involved in the VPF/VEGF-induced NRP-1-mediated EC migration signaling pathway, and again suggests that the effect of VPF/VEGF on EC mobility is mediated through the RhoA pathway at least in early passaged HUVECs (23). This conclusion is not in conflict with Liu et al’s result that overexpression of a dominant negative Rho did not inhibit VPF/VEGF-stimulated bovine pulmonary artery endothelial cell (BPAEC) migration (40). Possible reasons for the apparent inconsistency are that arterial ECs may have different physiological phenotypes (41), and that the early passaged (passage 3~4) HUVECs in our experiments may have a different pattern of expression of VPF/VEGF receptors from BPAECs of passage 19~24 used in their experiment. As Ly294002 (a PI-3K inhibitor) inhibits theVEGFR-1 signaling and therefore inhibits VPF/VEGF-stimulated migration of late passaged BPAEC (42), it is likely that VEGFR-1 may mediate VPF/VEGF-induced migration of late passaged BPAECs. NRP-1 stimulation leads to RhoA-dependent HUVEC migration was further identified by
RhoA activation assay in this study. With the results of pull-down assays, we found that EGNP-1 mediated EGF-induced RhoA activation in HUVECs but the mutant of EGNP-1, EGNP-1ΔSEA, did not showed the mediatory function of EGNP-1. Furthermore, We demonstrated PI-3K inhibitor (Ly294002) and the dominant negative mutant p85(DN) could inhibit this activation. Pretreatment with an antagonist of Gq family proteins, Gp Antagonist-2A, inhibits EGNP-1-mediated RhoA activation, which further confirms the involvement of Gq family proteins in NRP-1 signaling; Gβγ-sequestering peptide hβARK(495) inhibits EGNP-1-induced activation indicating that Gβγ is the upstream mediator of NRP-1 signaling to RhoA activation. Taken together, these results demonstrate that NRP-1-mediated RhoA activation requires Gq family proteins, free Gβγ subunits, and PI-3K activation. Overall, our current model for NRP-1 stimulated leads to EC migration is: VPF/VEGF → NRP-1 → PDZ domain-containing protein → Gq family proteins → Gβγ subunits → PI-3K → RhoA → EC migration. Our future studies will be aimed at elucidating more complete mechanisms of NRP-1 functional activity and identifying the functional activities in vivo.

A major contribution of this study is the findings that NRP-1 alone can mediate VPF/VEGF-induced EC migration, that the intracellular domain of NRP-1 is involved in this function, and that the C-terminal three amino acids are essential for this function. As invasion and metastatic spread is viewed as angiogenesis-dependent events (43), regulating EC migration and tumor cell migration as well as spread have drawn considerable attention from investigators interested in cancer control. The finding that NRP-1 alone can mediate EC migration, along with the fact that NRP-1 is not only expressed in EC but also in metastatic tumor cells (4), suggests that NRP-1 may be an important determinant of metastasis. Therefore, this study holds significant implications both in understanding the mechanisms of tumor cell metastasis and, potentially, in targeting NRP-1 for anti-cancer therapy.

**Acknowledgements:** This work was partly supported by grants from NIH (HL70567, HL072178 and CA78383), American Cancer Society and Department of Defence Breast Cancer Program to DM., and partly supported by grants from NIH (CA 45548) to SS.
References

Figure Legends

**Fig. 1. Construction and expression of the chimeric receptor EGNP-1 in HUVECs.** a) The extracellular domain of the EGFR was fused to the transmembrane and intracellular domains of NRP-1 and NRP-1ΔSEA to the creation of chimeric fusion receptor EGNP-1 and EGNP-1ΔSEA respectively; b) The extracts of HUVECs that had been transduced with LacZ and EGNP-1 respectively were immunoblotted with an antibody against the C-terminus of NRP-1 (Anti-NRP-1-C) (Left two lanes) or immunopecipitated with an antibody against the N-terminus of EGFR (Anti-EGFR-N) and Immunoblotting as above (Right two lanes); c) FACS analysis of HUVECs transduced with LacZ, EGDR and EGNP-1 respectively with fluorescent anti-EGFR-N or mouse IgG.

**Fig. 2. Effect of NRP-1/EGNP-1 signaling on HUVEC migration and proliferation.** a) Migration assay (I) and Proliferation assay (II) were carried out respectively in HUVECs (with 10 ng/ml VPF/VEGF stimulation) or HUVECs transduced with equal amounts of EGNP-1, EGDR, EGLT, as well as LacZ (with 10 ng/ml EGF stimulation). Immunoblotting (III) was carried out in these cells with anti-VEGFR-1-C, anti-VEGFR-2-C, and anti-NRP-1-C, respectively; to check the expression levels of EGLT, EGDR, and EGNP-1; b) HUVECs were transduced with varying amounts of EGNP-1. 10ng/ml EGF-induced migration was performed in these cells (I) and the related expression levels of EGNP-1 were also measured by immunoprecipitation with anti-EGFR-N and immunoblotting with anti-NRP-1-C (II).

**Fig. 3. NRP-1/EGNP-1 alone mediates HUVEC migration.** a) HUVECs transduced with EGNP-1 or co-transduced with EGNP-1 and EGDR (Y951F), EGDR (Y1059F), EGLT (793stop), EGLT (824stop), or LacZ were stimulated with 10 ng/ml EGF for migration assays (I). The expression of EGNP-1 was checked by Immunoblotting with anti-NRP-1-C (II). b) HUVECs and HUVECs transduced with EGNP-1 were treated with different concentrations of anti-VEGFR-2 (0, 1, 5 µg/ml), then were stimulated with 10 ng/ml VPF/VEGF or 10 ng/ml EGF for migration assays.
Fig. 4. Involvement of PI-3K in the NRP-1/EGNP-1 signaling pathway in HUVEC. a) Migration assay was carried out in HUVECs transduced with EGNP-1 which were treated with and without PI-3K inhibitor Ly294002 (25 µM) for 5 min and were then stimulated with 10 ng/ml EGF; b) HUVECs transduced with EGNP-1 or co-transduced with EGNP-1 and p110CAAX, p85(DN), as well as LacZ respectively, then the migration assay was performed with 10 ng/ml EGF stimulation (I) and immunoblotting was performed with anti-NRP-1-C (II).

Fig. 5. Involvement of G-protein and Gßγ subunits in NRP-1/EGNP-1-mediated HUVEC migration. a) Migration assay. HUVECs transduced with EGNP-1 were pretreated with and without Pertussis toxin (100 ng/ml) for 16 h or TATGFp (1 µM) for 5 min, then were stimulated with 10 ng/ml EGF; b) HUVECs were transduced with EGNP-1 or co-transduced with EGNP-1 and hßARK1(495) or LacZ. With these cells, the migration assay was carried out with 10 ng/ml EGF stimulation (I) and the immunoblotting was carried out with anti-NRP-1-C (II).

Fig. 6. Effect of small GTPase on NRP-1/EGNP-1-mediated HUVEC migration. HUVECs transduced with EGNP-1 or co-transduced with EGNP-1 and dominant negative mutants of CDC42-17N, Rac1-17N and RhoA-19N respectively were stimulated with 10 ng/ml EGF for the migration assay (I). Immunoblotting was carried out with anti-NRP-1-C to check the expression levels of EGNP-1 in these cells (II).

Fig. 7. NRP-1 signaling pathway and RhoA activation in HUVEC. RhoA activation assay was carried out. a) NRP-1/EGNP-1-mediated activation of RhoA. HUVECs transduced with LacZ or EGNP-1 were stimulated with 10 ng/ml EGF for different lengths of time (0, 0.5, 1, and 5 min); b) Effect of PI-3K on RhoA activation. (I) HUVECs transduced with EGNP-1 were incubated with and without PI-3K inhibitor Ly294002 (25 µM) for 5 min before addition of 10 ng/ml EGF for 1 min. (II) HUVECs co-transduced with EGNP-1 and LacZ or p85(DN) were stimulated with and without 10 ng/ml EGF for 1 min; c) Effect of G-protein and Gßγ subunits on RhoA activation. (I) HUVECs transduced with EGNP-1 were incubated with and without Pertussis toxin (100 ng/ml) for 16 h or TATGFp (1 µM) for 5 min before addition of 10 ng/ml EGF for 1 min. (II) HUVECs co-transduced with EGNP-1 and LacZ or hßARK1(495) were stimulated with and without 10 ng/ml EGF for 1 min.
Fig. 8. Effect of the C-terminal three amino acids of NRP-1 (S-E-A-COOH) on HUVEC migration. a. HUVECs were transduced with LacZ, EGNP-1 and EGNP-1ΔSEA. (I) The extracts of these cells were immunoblotted with anti-NRP-1-C (the left three lanes) or were immunoprecipitated with anti-EGFR-N and immunoblotted with anti-NRP-1-C (the right three lanes). (II) FACS analysis the cell surface expression of EGNP-1-SEA in these cells with fluorescent anti-EGFR-N or mouse IgG; b) HUVECs or HUVECs transduced with LacZ, EGNP-1, and EGNP-1ΔSEA were stimulated with 10 ng/ml VPF/VEGF or 10ng/ml EGF for migration assay (I). Immunoblotting was carried out with anti-NRP-1-C to check the expression levels of the target proteins in these cells (II); c). Effect of EGNP-1ΔSEA on RhoA activity. HUVECs transduced with EGNP-1ΔSEA were stimulated with 10ng/ml EGF for different lengths of time (0, 0.5, 1 and 5 min).
**Fig. 1a**

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<td>1-640/856</td>
<td>920-923</td>
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**Fig. 1b**

Transductions

- LacZ
- EGNP-1

IB: Anti-NRP-1-C (cytoplasmic)

IP: Anti-EGFR-N (extracellular)
Fig. 1c

LacZ/anti-EGFR-N

EGNP/anti-EGFR-NEGNP/mouse IgG

EGDR/anti-EGFR-N

EGNP/anti-EGFR-N
**Fig. 2a**

III

Transductions

LacZ  
EGLT

IB: Anti-VEGFR-1-C

LacZ  
EGDR

IB: Anti-VEGFR-2-C

LacZ  
EGNP-1

IB: Anti-NRP-1-C

**Fig. 2b**

I

<table>
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<tr>
<th>EGNP-1 (ml)</th>
<th>0</th>
<th>0.5</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
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<tbody>
<tr>
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<td></td>
<td></td>
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</tr>
</tbody>
</table>

II

Transductions of EGNP-1 (ml)

0  
0.5  
1  
2  
3  
4  

EGNP-1

IP: Anti-EGFR-N

IB: Anti-NRP-1-C
**Fig. 3a**

**I**

![Graph showing cell migration](image)

- **No. of Migrating Cells**
- **Y-axis**
  - 0
  - 5000
  - 10000
  - 15000
  - 20000
  - 25000
  - 30000
  - 35000

- **X-axis**
  - EGNP-1/LacZ
  - EGNP-1/EGDR
  - EGNP-1/EGDR(Y951F)
  - EGNP-1/EGDR(Y1059F)
  - EGNP-1/EGLT(793stop)
  - EGNP-1/EGLT(824stop)
  - EGNP-1/EGLT(824stop)

- **Legend**
  - Open square: unstimulated
  - Filled square: EGF (10 ng/ml)

**II**

- **Transductions**
  - EGNP-1
  - EGNP-1/LacZ
  - EGNP-1/EGDR
  - EGNP-1/EGDR(Y951F)
  - EGNP-1/EGDR(Y1059F)
  - EGNP-1/EGLT

- **Western Blot**
  - NRP-1
  - EGNP

**IB:** Anti-NRP-1-C
Fig. 4b

I

![Bar graph showing the number of migrating cells with and without EGF stimulation.]

- EGNP-1
- EGNP-1/LacZ
- EGNP-1/p85(DN)
- EGNP-1/p110CAAX

unstimulated
EGF (10 ng/ml)

II

![Western blot with IB: Anti-NRP-1-C showing transduction effects.]

Transductions: EGNP-1
- EGNP-1/LacZ
- EGNP-1/p85(DN)
- EGNP-1/p110CAAX

Fig. 5a

![Bar graph showing the number of migrating cells with different treatments.]

- EGNP-1
- EGNP-1
- EGNP-1

- Pertussis toxin (100 ng/ml)
- TATGFp (1 µM)

unstimulated
EGF (10 ng/ml)
Fig. 5b

I

![Graph showing the number of migrating cells with different transductions.](image)

II

![Western blot analysis showing NRP-1 and EGNP-1 expression with different transductions.](image)
Fig. 6

I

![Bar graph showing the number of migrating cells with and without EGF stimulation.](image)

- **EGP-1**
- **EGP-1/LacZ**
- **EGP-1/CDC42-17N**
- **EGP-1/Rac1-17N**
- **EGP-1/RhoA-19N**

- **No. of Migrating Cells**

- **Legend:**
  - □ unstimulated
  - ▼ EGF (10 ng/ml)

II

<table>
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<tr>
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<th>EGP-1</th>
<th>EGP-1/LacZ</th>
<th>EGP-1/CDC42-17N</th>
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IB: Anti-NRP-1-C
**Fig. 7a**

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<tr>
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<th>EGF (10 ng/ml)</th>
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<tr>
<td></td>
<td>0</td>
<td>0.5</td>
<td>1</td>
<td>5 min</td>
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<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>EGFP-NP-1</td>
<td>-</td>
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Activated RhoA

Cellular RhoA

**Fig. 7b**

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<tr>
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<tr>
<td>p85(DN)</td>
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Activated RhoA

Cellular RhoA

**Fig. 7c**

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<tr>
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<tr>
<td>LacZ</td>
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<tr>
<td>hßARK1(495)</td>
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</table>

Activated RhoA

Cellular RhoA
Fig. 8a

I

Transductions

LacZ  EGNP-1  EGNP-1/SER  LacZ  EGNP-1  EGNP-1/SER

NRP-1  EGNP-1

IB  IP + IB  EGNP-1

IP: Anti-EGFR-N
IB: Anti-NRP-1-C

Fig. 8a

II

LacZ/anti-EGFR-N  EGDR/anti-EGFR-N

EGNP-1/SER/001  EGNP-1/SER/003

Counts

0 10 100 1000 10000 100000 1000000 10000000

FL1-H

EGNP-1/SER/002  EGNP-1/SER/004

Counts

0 20 40 60 80 100

FL1-H
Fig. 8b

I

![Bar graph showing the number of migrating cells](image)

- Non-transfected
- LacZ
- EGNP-1
- EGNP-1?SEA

Legend:
- □ unstimulate
- ■ VPF/VEGF (10ng/ml)
- ▣ EGF (10 ng/ml)

II

Transductions
- Non-transfected
- LacZ
- EGNP-1
- EGNP-1?SEA

IB: Anti-NRP-1-C

Fig. 8c

![Western blot showing cellular and activated RhoA](image)

- Transduction
- EGNP-1?SEA
- Cellular RhoA
- Activated RhoA

EGF (10 ng/ml) 0 0.5 1 5 min
Neuropilin-1-mediated vascular permeability factor / vascular endothelial growth factor (VPF/VEGF)-dependent endothelial cell migration

Ling Wang, Huiyan Zeng, Ping Wang, Shay Soker and Debabrata Mukhopadhyay

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