VEGF-A_{165} Induces Human Aortic Smooth Muscle Cell Migration by Activating Neuropilin-1-VEGFR1-PI3K Axis†

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ABSTRACT: Vascular smooth muscle cells (SMCs), one of the major cell types of the vascular wall, play a critical role in the process of angiogenesis under both physiological and pathophysiological conditions, including the cancer microenvironment. Previous studies have shown that VEGF-A_{165} augments vascular SMC migration via VEGFR2 (KDR/Flk1) pathways. In this study, we found that VEGF-A_{165} (recombinant protein or breast tumor cell-secreted) is also capable of inducing migration of VEGFR2-negative human aortic smooth muscle cells (hAOSMCs), and this induction is mediated through a molecular cross-talk of neuropilin-1 (NRP-1), VEGFR1 (Flt-1), and phosphoinositide 3-kinase (PI3K)/Akt signaling kinase. We found that VEGF-A_{165} induces hAOSMC migration parallel with the induction of NRP-1 and VEGFR1 expressions and their associations along with the activation of PI3K/Akt. Neutralization of VEGF action by its antibody or inhibition of VEGF-induced PI3K/Akt kinase activation by wortmannin, a PI3K/Akt specific inhibitor, results in inhibition of VEGF-induced hAOSMC migration. Moreover, RNAi-mediated elimination of the NRP-1 expression or blocking of the activity of VEGFR1 by its antibody in hAOSMCs impairs the VEGF-A_{165}-induced migration of these cells as well as activation of PI3K/Akt kinase. Collectively, these results establish, for the first time, a mechanistic link among VEGF-A_{165}, NRP-1, VEGFR1, and PI3K/Akt in the regulation of migration of human vascular smooth muscle cells that eventually could be involved in the angiogenic switch.

The molecular interactions between cells are indispensable measures of the maintenance of physiological (*1*) and pathophysiological processes (*2*), including tumor angiogenesis or neovascularization, which is required for tumor growth and metastasis (*3, 4*). During angiogenesis, vascular smooth muscle cells (SMCs), the middle layer of blood vessels, play an important role in constructing a new blood vessel by migrating from roots to tips of newly formed capillaries (angiogenic sprouting) (*3, 5*). Migration of vascular SMCs is a complex, multistep process, and cell−cell interactions including tumor cells are required to accomplish this event (*6*). However, very little is known about molecular steps associated with this event.

Various tumor cells, including breast tumor cells, secrete excess proangiogenic growth factors for tumor vascularization. Among the known angiogenic factors, vascular endothelial growth factor-A (VEGF-A)[1] has emerged as the central regulator of angiogenesis (*7–12*). VEGF-A is a secreted homodimeric glycoprotein and a member of the VEGF family of ligands of three important tyrosine kinase family receptors called VEGFR1 (Flt-1), VEGFR2 (Flk-1/KDR), and VEGFR3 (Flt-4) (*13–16*). There are four alternative spliced variants of VEGF-A, including VEGF-A_{206}, VEGF-A_{189}, VEGF-A_{165}, and VEGF-A_{121}, with VEGF-A_{165} being the prime isoform (*17, 18*). During physiological or pathobiological angiogenesis, VEGF-A acts as a chemoattractant and mitogen for endothelial cells (ECs), which ultimately form the endothelial backbone of a vessel (*19–22*). This activity of VEGF-A is primarily exerted when it binds with its prime receptor VEGFR2 which is then followed by the activation of multiple signaling pathways in ECs. Moreover, neuropilin-1 (NRP-1), a type 1 transmembrane protein (*23, 24*) and coreceptor of VEGF-A_{165} (*25*), also participates in the migration of ECs by enhancing the binding of VEGF-A_{165} with VEGFR2 (*25*). Like ECs, vascular SMC migration can be achieved by VEGF-A (*26, 27*). However, the precise mechanisms that are involved in VEGF-A-induced migration of vascular SMC are incomplete and complex. Previous

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[1] Abbreviations: hAOSMCs, human aortic smooth muscle cells; shRNA, short-hairpin RNA; NRP-1, neuropilin-1; RNAi, RNA interference; VEGF, vascular endothelial growth factor; VEGFR1, vascular endothelial growth factor receptor 1; PI3K, phosphoinositide-3 kinase.

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studies have demonstrated that the enhanced migration of human vascular smooth muscle cells may be mediated through its receptors (*26*). Since then, Liu et al. (*28*) have reported a similar finding, which indicates that b-FGF-induced VEGF may be mediated through the binding of VEGF-A_{165} to both NRP-1 and VEGFR2. In contrast, other studies demonstrated an indirect effect of VEGF-A on vascular SMC migration (*27*). In this report, we found that VEGF-A_{165} (tumor cell-secreted or recombinant protein) cross-talks with VEGFR1 and NRP-1 to activate the PI3K/Akt signaling molecule in VEGFR2 deficient hAOSMCs to enhance the migration of these cells.

MATERIALS AND METHODS

Cell Culture. Breast tumor cells (i.e., MCF-7 and MDA-MB-231) were obtained from American Type Culture Collection (Manassas, VA). hCASMCs (human coronary artery smooth muscle cells), hAOSMCs (human aortic smooth muscle cells), and nontransformed human mammary epithelial cells (HMECa) were obtained from Cambrex Bio Science Inc. (Walkersville, MD). hAOSMCs and hCASMCs were maintained in defined medium (DM) and contain smooth muscle basal medium (SmBM) with various growth factors (i.e., insulin, FGF, and EGF) and 2% serum. HMECs were grown in mammary epithelial basal medium with growth factors. Media for vascular SMCs and HMECs and growth factors were purchased from Cambrex Bio Science Inc. MCF-7 and MDA-MB-231 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma Chemical Co., St Louis, MO) and supplemented with 10% fetal bovine serum and antibiotics. Cells were used for the experiment between four and six passages.

Reagents. VEGF-A_{165} (recombinant protein), wortmannin, and Giemsa powder were obtained from Sigma Chemical Co. Trizol, Lipofectin, and Opti-MEM were purchased from Life Technologies (Grand Island, NY). Polyclonal anti-rabbit VEGF antibody was purchased from Zymed Laboratories Inc. (San Francisco, CA). Rabbit anti-VEGF receptor 1 polyclonal antibody and mouse anti-VEGF receptor 2 monoclonal antibody were purchased from Laboratory Vision (Fremont, CA) and abcam (Cambridge, MA), respectively. Rabbit antineuropilin-1 polyclonal antibody was a gift from Dr. Kolodkin. The RT-PCR RNA amplification kit was obtained from Applied Biosystems (Branchburg, NJ). U0126 was purchased from Promega (Madison, WI).

Preparation of Conditioned Media. To make the conditioned medium (CM), tumor cells were grown in defined medium for 24 h. The media were collected by centrifugation and stored at 4 °C until they were used for the migration.

Immunoprecipitation. The immunoprecipitation assay was performed according to our previous method (*29*). Briefly, cells were lysed in lysis buffer [50 mM Tris-HCl (pH 8.0), 150 μM NaCl, 1% Triton X-100, and protease inhibitor cocktail], and protein concentrations were measured using the Coomassie blue reagent assay (*30*). Protein (1 mg/mL) was incubated with Protein A−agarose beads for 1 h at 4 °C followed by overnight incubation with the appropriate antibody. The immunoprecipitated protein was boiled and separated by SDS−PAGE and transferred to a nitrocellulose membrane. Signals were detected by Immuno-Western blot analysis using specific antibodies.

Immuno-Western Blot Analysis. Equal amounts of protein (30 μg) were subjected to 7.5 or 10% SDS−PAGE, and the gel-fractionated proteins were transferred to a nitrocellulose membrane for the detection of different proteins by specific antibodies. Signals were detected with Super Signal ULTRA chemiluminescent substrate (Pierce, Rockford, IL) by using one-dimensional Image Analysis, version 3.6 (Eastman Kodak Co., Rochester, NY).

RT-PCR Analysis. Cytoplasmic RNAs were extracted from SMC using the Trizol extraction procedure (*31*) and were subjected to reverse transcription using a RT-PCR RNA amplification kit (Perkin-Elmer). The cDNAs for human NRP-1, VEGFR2, and GAPDH (for equal loading) were PCR amplified with specific primers. PCR primers for NRP-1 and GAPDH were the same as those described previously (*29*). The sequences of the primers of VEGFR2 were 5′-CCTCTGTGGGTTTGCCTAGT-3′ (forward) and 5′-CAC-GACTCCATGTTGGTCAC-3′ (reverse).

Short Hairpin RNA (shRNA) Synthesis, Cloning, and Transfection. The shRNA experiments are the same as those described previously (*29*). Briefly, two different target sites of human NRP-1 cDNA sequences and mismatched sequence were selected for shRNAs, chemically synthesized (Midland Certified Reagent Co., Midland, TX), and cloned into the pSilencer 1.0-U6 expression vector. The shRNAs sequences of human NRP-1 were the same as those described previously (*29*). The mismatch shRNA was used in this study as a negative control. shRNAs were transfected into AOSMCs using the Lipofectin Reagent (Invitrogen). After transfection for 48 h, AOSMCs were used for the migration assay.

Migration Assay. The chemotaxis assay was conducted using a modified Boyden chamber technique as described previously (*29*). Briefly, vascular SMCs (20000 cells/well) were added to the upper chambers of the Boyden chambers containing SmBM with VEGF recombinant protein (50 ng/mL) in the lower chamber and incubated for 8 h at 37 °C in a CO_2 incubator. The migrated cells were fixed with methanol, stained with a 2% Giemsa solution, and counted in 15–20 randomly chosen fields for each sample.

Neutralizing Assay. The conditioned medium derived from semiconfluent MCF-7 cells was preincubated with polyclonal anti-VEGF antibody at different concentrations (i.e., 100, 200, and 500 ng/mL) to neutralize overnight at 4 °C. AOSMC migration was evaluated using the modified Boyden chamber with defined medium, tumor cell-conditioned medium, and neutralizing medium. The migration assay was performed after incubation for 8 h, and the cells were fixed, stained, and counted.

Statistical Analyses. All experiments were performed in triplicate. Each of the datum represents the mean ± standard error from two separate experiments. Statistical analysis was performed between the two groups of data by an unpaired Student's t test. A p value of <0.05 was considered statistically significant.

RESULTS

Breast Tumor Cell-Generated VEGF-A_{165} Protein Enhances Human Aortic Smooth Muscle Cell Migration. Our goal in this study was to establish whether tumor cell-generated VEGF-A_{165} is able to induce the migration of human vascular SMCs. To test this, first we determined the

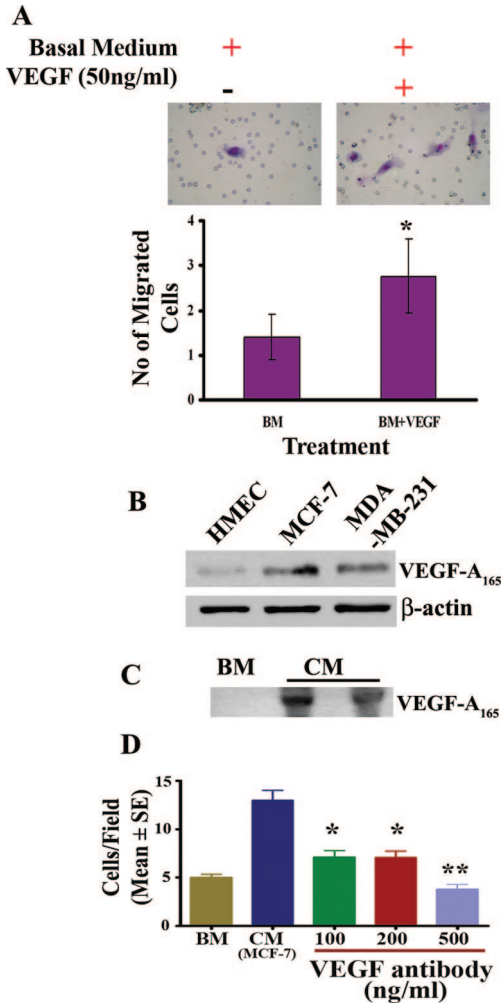

FIGURE 1: Effect of VEGF-A_{165} on hAOSMCs migration. (A) Microphotographs illustrate the migration of hAOSMCs in the presence or absence of VEGF-A_{165}. The bar graph shows the number of migrated cells under different culture conditions. (B and C) Western blot analysis of VEGF-A_{165} expression in normal and different breast tumor cell lysates and in their media, respectively. The top panel shows the expression of VEGF-A_{165} protein in HMEC, MCF-7, and MDA-MB-231 breast tumor cells lysates, and the bottom panel shows the expression level of VEGF-A_{165} (secreted) protein in the basal and conditioned media of MCF-7 and MDA-MB-231 cells. BM stands for basal medium and CM for conditioned medium. (D) Results of the neutralization assay. The neutralization was carried out by using different doses of VEGF-A antibodies for 8 h as described in Materials and Methods. Data are displayed as means ± the standard error of at least three independent experiments. *p* values were determined by a Student's *t* test. One asterisk indicates a *p* of <0.01 vs untreated or CM; two asterisks indicate a *p* value of <0.001 vs CM.

effect of recombinant VEGF-A_{165} on the migration of human aortic smooth muscle cells (hAOSMCs). To do so, the semiconfluent hAOSMCs were seeded on the upper well of a modified Boyden chamber containing SMC's basal medium (without growth factors or serum); human recombinant VEGF-A_{165} (50 ng/mL) protein was added to the lower wells of the chambers for 8 h at 37 °C, and the migration of these cells through the membrane was evaluated. As shown in Figure 1A, recombinant VEGF-A_{165} is able to stimulate the migration of hAOSMCs significantly as compared to vehicle-treated basal medium. Consistent results were found in CASMCs (data not shown).

Next we sought to determine whether tumor cell-generated VEGF-A_{165} protein could enhance vascular SMC migration. To test this, we prepared conditioned medium from a normal mammary epithelial cell culture (HMEC), a noninvasive breast tumor cell culture (MCF-7), and a highly aggressive breast tumor cell culture (MDA-MB-231). The status of VEGF-A was evaluated in these cell lines and in the conditioned media by Western blot analysis using a polyclonal antibody that immunoreacts specifically with VEGF-A_{165} and VEGF-A_{206}. The level of VEGF protein, recognized as a single band of 24 kDa protein, was strikingly higher in MCF-7 as well as in MDA-MB-231 cells and their conditioned media than in HMECs or basal media (Figure 1B,C). VEGF antibody exhibited fewer or no immunoreactions in HMEC lysates. Since the VEGF-A_{165} positive control band and the immuno-reacted band in tumor cell extracts migrate an equal distance in SDS−PAGE, the identified band in tumor cell extracts can be considered VEGF-A_{165} protein (data not shown). Collectively, this study demonstrates that a significant amount of VEGF-A_{165} is produced and secreted by MCF-7 and MDA-MB-231 breast tumor cells. We next looked for the effect of secreted VEGF-A_{165} from tumor cell-conditioned media on migration of hAOSMCs. To test this, hAOSMCs were exposed to regular defined (unconditioned) medium, MCF-7-conditioned medium, or conditioned medium pretreated with a neutralizing antibody against VEGF-A. The level of migration of hAOSMCs in MCF-7 cell-conditioned medium was increased by 3.5-fold compared to that in basal medium. When hAOSMCs were grown in neutralizing antibody-pretreated conditioned medium for 8 h, the level of migration of the hAOSMCs was significantly reduced compared to that of the non-neutralized conditioned medium (Figure 1D). The inhibitory effect of VEGF-A antibody was detected at a concentration of 100 ng/mL. The maximum effect was detected at a dose of 500 ng/mL (recommended by the company). Nonimmune rabbit serum showed no detectable amount of effect on hAOSMC migration. To rule out the apoptotic or antiproliferatic effect of neutralizing antibody-pretreated conditioned media on an apparent reduction in the level of cell migration, we also determined cell viability using the MTT assay. We found that the pretreated conditioned medium was unable to exhibit a catastrophic effect on these cells after a short (8 h) exposure (data not shown).

Status of VEGF-A Receptors and the Coreceptor in Human AOSMCs. The expressions and functions of two important VEGF-A receptors (i.e., VEGFR1 and VEGFR2) have been studied predominantly in endothelial cells from various sources, including human tissues and cells (*14*), but their expressions have also been assessed in some non-human vascular SMCs (*26, 27, 32*). Because the status of VEGFR1 and VEGFR2 has not yet been explored in human AOSMCs, we analyzed whether hAOSMCs expressed VEGFR-1 and VEGFR2 as well as NRP-1, a coreceptor for VEGF-A_{165}, by reverse-transcribed polymerase chain reaction (RT-PCR), Western blot analysis, or both. We found that VEGFR1 protein was expressed in hAOSMCs (Figure 2A, left panel) but VEGFR2 mRNA or protein expression was undetected in these cells (Figure 2B, left and right panels). NRP-1 transcript and protein were also found to be expressed in hAOSMCs (Figure 2C, left and right panels), which is consistent with our previous work (*29*). Furthermore, we

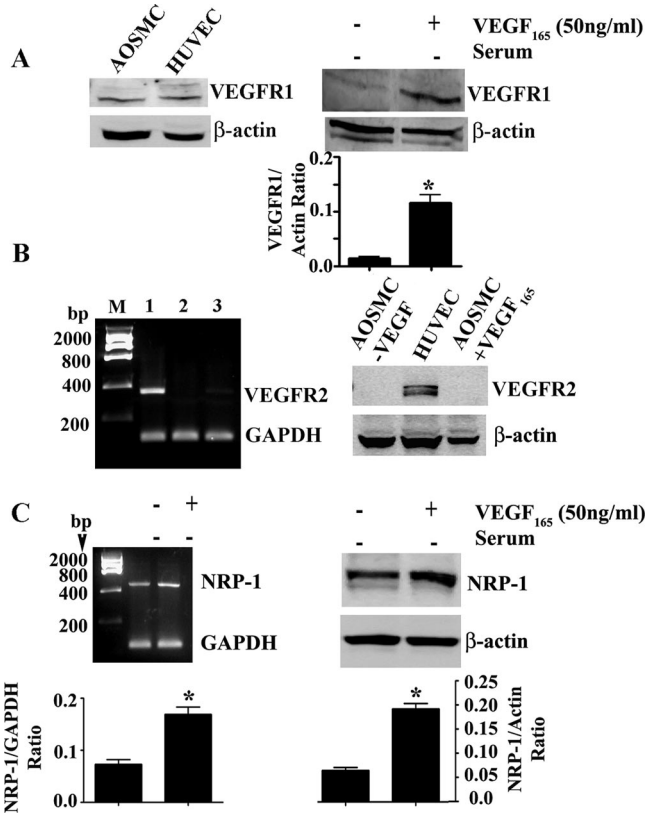

FIGURE 2: Effect of VEGF-A_{165} on VEGFR1/Flt-1, VEGFR2/Flk-1, and NRP-1 mRNA and protein expression in hAOSMCs. (A) Representative blots showing the expression of VEGFR1 protein in hAOSMCs and HUVECs (left panel) and the effect of VEGF-A_{165} recombinant protein on VEGFR1 expression in hAOSMCs (right panel). (B) Representative photographs of RT-PCR (left panel) and Western blot (right panel) showing the level of VEGFR2 in hAOSMCs before and after VEGF-A_{165} recombinant protein exposure. HUVEC lysate was used as a positive control. Lane 1 shows the mRNA status of HUVEC (positive control), lane 2 untreated hAOSMCs, and lane 3 VEGF-treated (50 ng/mL) hAOSMCs. (C) NRP-1 mRNA and protein level detected in VEGF-A_{165}-exposed or unexposed hAOSMCs using RT-PCR and Western blot analysis, respectively. Data are displayed as means ± the standard error of at least three independent experiments. *p* values were determined by a Student's *t* test. An asterisk indicates a *p* value of <0.01 vs untreated.

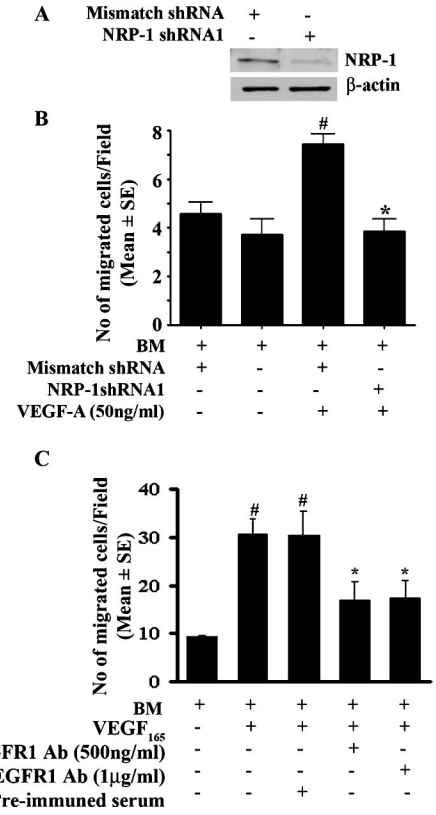

FIGURE 3: Effects of NRP-1 specific shRNA and VEGFR1 neutralizing antibody on VEGF-A_{165}-induced migration of AOSMCs. (A) This photograph illustrates the effect of NRP-1 specific shRNA on NRP-1 protein production in hAOSMCs. (B) Bar graph showing the impact of VEGF-A_{165} on the migration of NRP-1 shRNA-transfected AOSMCs. Data are displayed as means ± the standard error of three independent experiments. A number sign indicates a *p* value of <0.005 vs controls, and an asterisk indicates a *p* value of <0.01 vs VEGF-treated. (C) Bar graph showing the effect of VEGFR1 neutralizing antibody on VEGF-A_{165}-dependent AOSMC migration. Data are displayed as means ± the standard error of at least three independent experiments. A number sign indicates a *p* value of <0.001 vs controls, and an asterisk indicates a *p* value of <0.01 vs VEGF-treated.

found that the treatment of AOSMCs with recombinant VEGF-A_{165} protein (50 ng/mL) under serum free conditions for 8 h augmented NRP-1 and VEGFR1 expression by 1.8- and 7.0-fold, respectively, without inducing the VEGFR2 mRNA or protein (Figure 2B). SDS−PAGE analysis of 100 μg or larger quantities of proteins failed to reveal an increase in VEGFR2 levels (not shown). These data indicate that NRP-1 and VEGFR1 may be the key players in VEGF-A_{165}-dependent migration of hAOSMCs, while VEGFR2, although required for VEGF-mediated EC migration (*33*), is unlikely to be involved in these processes.

Effect of NRP-1 Silencing on VEGF-A_{165}-Induced AOSMC Migration. To determine whether NRP-1 is indispensable for VEGF-A_{165}-induced migration of hAOSMCs, NRP-1 expression was repressed in hAOSMCs using shRNA and then VEGF-A_{165}-dependent migration was evaluated. First, we tested the efficacy of the shRNAs, selected for this study, in silencing of NRP-1 expression. We found shRNA-1 effectively blocked NRP-1 expression while the mismatched shRNA had no effect on NRP-1 expression in these cells (Figure 3A). Like shRNA-1, shRNA-2 also caused NRP-1 depletion (data not shown). Next, we analyzed whether NRP-1 silencing blocked VEGF-A_{165}-induced hAOSMC migration. We found VEGF-A_{165} was unable to enhance the migration of NRP-1-depleted vascular SMCs while mismatched shRNA had no effect on VEGF-A_{165}-induced migration (Figure 3B). Knocking down the level of NRP-1 expression did not interfere with proliferation of hAOSMCs (data not shown). Together, these data show that VEGF-induced migration is NRP-1-dependent.

Effect of VEGFR1 Neutralizing Antibody on VEGF-A_{165}-Induced AOSMC Migration. To examine whether VEGFR1 participates in VEGF-A_{165}-induced AOSMC migration, we blocked the VEGFR1 activity by treating the cells with a neutralizing antibody specific for VEGFR1 or preimmune serum for 24 h. The cells were then seeded in the upper chamber of the Boyden chamber for in vitro migration analysis. The studies revealed that the VEGFR1 antibody-mediated inhibition of VEGFR1 action significantly impairs the VEGF-induced AOSMCs migration (Figure 3C), while this effect was undetected in cells exposed to preimmune serum.

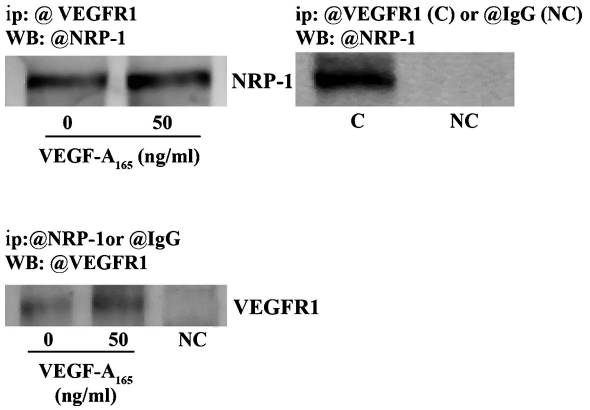

FIGURE 4: Detection of physical interaction of VEGFR1 with NRP-1 in AOSMCs. Cell lysates (VEGF-treated or untreated) were immunoprecipitated with either VEGFR1 or NRP-1 antibodies or IgG (negative control). VEGFR1 or NRP-1 protein was detected by immuno-Western blotting. C, untreated control; NC, negative control.

Physical Interactions of NRP-1 and VEGFR1 in AOSMCs. According to the observations, AOSMCs express VEGFR1 and NRP-1 but not VEGFR2, and both NRP-1 and VEGFR1 actively participate in VEGF-A-induced AOSMC migration (Figures 2 and 3). We, therefore, investigated whether NRP-1 and VEGFR1 physically interact with each other in these cells and whether VEGF-A has the ability to enhance their interaction. The immunoprecipitation followed by immuno-Western blot analysis with appropriate antibodies revealed that NRP-1 strongly interacts physically with VEGFR1, and this interaction can be enhanced by addition of recombinant VEGF-A_{165} protein to the culture medium (Figure 4). NRP-1 or VEGFR1 cannot be detected by immuno-Western blot analysis in the immunoprecipitated samples obtained with IgG (Figure 4).

AKT/PI3K Signaling Is Required for VEGF-NRP-1-Induced hAOSMC Migration. To determine which signaling pathways participated in VEGF-A_{165}-induced migration, chemotaxis assays were set up using the modified Boyden chamber technique. Human AOSMCs were exposed to wortmannin (100 nM), an Akt/phosphatidylinositol 3-kinase (PI3K) inhibitor, or U0126 (10 μM), a MAPK kinase/extracellular signal-regulated kinase inhibitor, for 30 min before being exposed to VEGF-A_{165} (50 ng/mL) for 8 h. As shown in Figure 5A, wortmannin was able to inhibit significantly VEGF-induced hAOSMC migration, while U0126 had no effect on VEGF-induced migration. Therefore, this study suggests that the PI3K/Akt signaling pathway is involved in VEGF-A_{165}-induced migration of human AOSMCs.

Next we determined whether NRP-1 is involved in VEGF-A_{165}-mediated activation of Akt/PI3K. To do so, NRP-1 was knocked down in hAOSMCs by shRNA as described above and then treated with recombinant VEGF-A_{165} for 8 h. Following treatment, the phospho-Akt and total Akt levels were determined by immuno-Western blot analysis using specific antibodies. There were no major differences in the expression profile of p-Akt in mismatch shRNA- and NRP-1 shRNA-transfected cells (Figure 5B). The level of expression of p-Akt was significantly higher ($p < 0.005$) in VEGF-treated mismatch shRNA-transfected hAOSMCs. However, the inductive effect of VEGF on p-Akt was not detected in

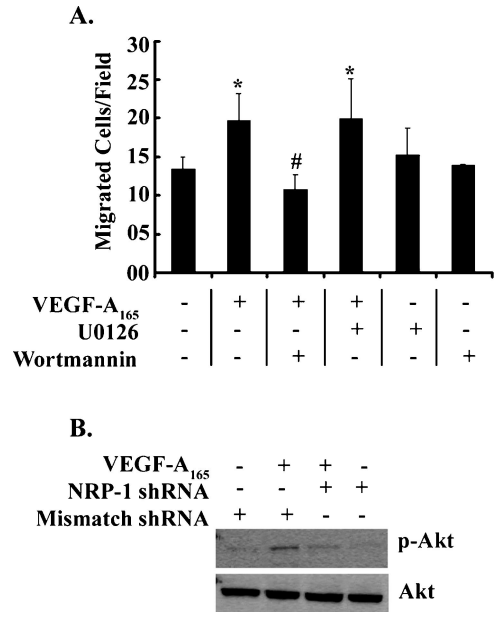

FIGURE 5: Involvement of the Akt signal transduction pathway in VEGF-A_{165}-VEGFR1-NRP-1-induced migration of hAOSMCs. (A) Analysis of the effect of VEGF on migration of vascular smooth muscle cells in the modified Boyden chamber in the presence or absence of an Akt inhibitor (wortmannin, 100 nM) or extracellular signal-regulated kinase inhibitor (U0126, 10 μM). Columns show the means of three different experiments. p values were determined by a Student's t test. An asterisk indicates a p value of <0.01 vs controls, and a number sign indicates a p value of <0.01 vs VEGF-treated samples. (B) Western blot analysis of the p-Akt protein level in VEGF-A_{165}-exposed NRP-1 knockout hAOSMCs or parental hAOSMCs.

NRP-1 shRNA-transfected cells (Figure 5B). Thus, the VEGF-induced activation of the Akt/PI3K signaling pathway is NRP-1-VEGFR1-dependent in hAOSMCs.

DISCUSSION

This study identifies a novel layer in the regulation of vascular smooth muscle cell migration under the influence of VEGF-A_{165} cytokine. We demonstrate that VEGF-A_{165} induces vascular SMC migration through the activation of the NRP-1-VEGFR1-PI3K/Akt signaling cascade in a VEGFR2-deficient environment. Moreover, the proposed molecular pathway is also relevant in breast tumor cell-induced migration of vascular SMCs, which is ultimately required for the activation of tumor angiogenesis and promotion of the maturation of new blood vessels (Figure 6).

Angiogenic switch, or new blood vessel formation from the pre-existing one, is required for tumor growth and metastasis. New blood vessel formation follows several steps, such as vasodilation and permeability, vessel destabilization, and matrix degradation, endothelial cell as well as vascular SMC proliferation and migration, and finally lumen formation and vessel stabilization (*3, 6*). Among all the processes, the mobilization of vascular SMCs is one of the important steps in angiogenesis for the stabilization of blood vessels (*3*). Previously, independent studies from different laboratories, including ours, have shown that tumor cells can induce the motility of vascular SMCs, and this process is controlled by various angiogenic factors, including VEGF-A (*26, 28, 29*). The findings presented here agree with the previous studies and propose that both recombinant VEGF-A_{165} and human

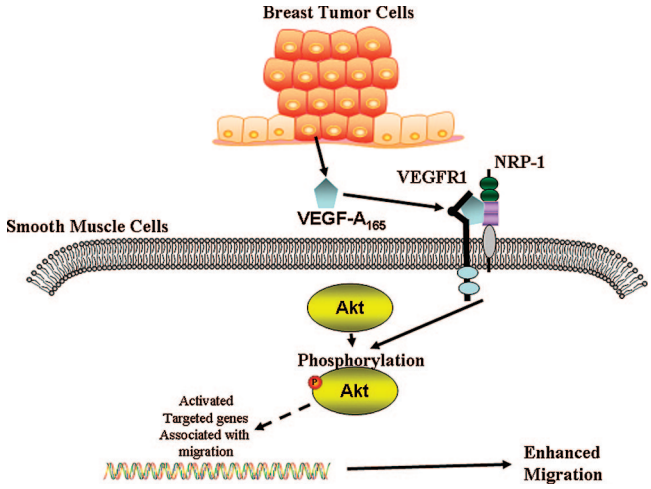

FIGURE 6: Diagrammatic illustrations of signaling pathways involved in VEGF-A_{165}-induced human aortic smooth muscle cell migration. The studies demostrate that VEGF-A_{165} induces vascular SMC migration through the activation of the NRP-1-VEGFR1-PI3K/Akt signaling cascade in a VEGFR2-deficient environment.

breast tumor cell (i.e., MCF-7 and MDA-MB-231)-secreted VEGF-A_{165} can enhance the migration of hAOSMCs (Figure 1A).

The means by which VEGF-A_{165} triggers the necessary signals required for the migration of hAOSMCs have remained a puzzling gap in understanding the mechanism of vascular SMC migration induced by VEGF-A_{165}. The studies suggest that the chemoattractant activity of VEGF on vascular SMCs could be mediated through its receptors (*26*), particularly VEGFR2 (*28*). However, our studies indicate that VEGF-A_{165} is capable of inducing hAOSMC migration in the absence of VEGFR2, because VEGFR2 protein is absent in these cells, and VEGF-A_{165} is unable to upregulate this protein level in hAOSMCs (Figure 2A,B). In contrast, VEGFR1 protein is found to be expressed in these cells and can be upregulated by VEGF-A_{165} recombinant protein (Figure 2A). We, therefore, anticipated the involvement of VEGFR1 in this event. Indeed, our studies have supported the hypothesis as blocking the activity of VEGFR1 in AOSMCs by VEGFR1 neutralizing antibody prevents chemoattractant activity of VEGF-A_{165} on vascular SMCs (Figure 3C).

NRP-1 is overexpressed in vascular SMCs of the blood vessels of human breast tumor samples (*34*) and also required for endothelial cell migration (*5, 33*) as well as PDGF-induced vascular SMC migration (*29*). We show here that NRP-1 is expressed in hAOSMCs, and recombinant VEGF-A_{165} protein increased its level of expression in both mRNA and protein level significantly parallel with the induction of mobilization of hAOSMCs (Figure 2C). The elimination of the expression of NRP-1 by NRP-1 specific shRNA resulted in inhibition of the VEGF-A_{165}-induced migration of hAOSMCs after transient transfection with a recovery of the post-transfection level by 48 h (Figure 3A). In addition, we also found that NRP-1 physically interacts with VEGFR1 in AOSMCs and this molecular interaction can be strengthened in these cells by exposing them to the recombinant VEGF-A_{165} protein (Figure 4). Thus, we suggest, unlike the case in endothelial cells (*35*), the molecular cross-talk between NRP-1 and VEGFR1 is adequate to result in induction of hAOSMC migration by VEGF-A_{165}. Our finding differs from those obtained in non-human vascular smooth muscle cells (*26*) or other types of smooth muscle cells (*28*), which anticipated an active involvement of VEGFR2 (KDR) in VEGF-induced migration of SMCs. This finding does not necessarily conflict with our data, because those experiments were conducted in vascular SMCs where VEGFR2 expression was abundant. On the basis of these studies, therefore, we could anticipate that both pathways could be involved in vascular SMC migration depending on the cellular microenvironment.

The PI3K/Akt signaling pathway is utilized by VEGF for its survival promoting activity as well as migration of endothelial cells (*36–44*). This signaling kinase is also activated by various stimulants for the proliferation and migration of vascular smooth muscle cells (*45–48*). Our pharmacological inhibitor experiments identify PI3K/Akt as an essential downstream signaling molecule in VEGF-A_{165}-induced hAOSMC migration. This study also shows that the activation of PI3K/Akt by VEGF-A_{165} in hAOSMCs is mediated through NRP-1 because silencing of the NRP-1 expression by shRNA inhibited the VEGF-induced activation of PI3K/Akt and eventually migration of hAOSMCs.

In summary, we have been able to demonstrate a novel mechanism by which VEGF-A_{165} enhances the migration of human vascular SMCs, and NRP-1 is an indispensible player in this event. Moreover, this and other studies suggest that the different cognate receptors may be exploited opportunistically by VEGF-A_{165}, depending on the microenvironment, in acquiring chemoattractant power on vascular SMCs.

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