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Expression of VEGFR-2 on HaCaT cells is regulated by VEGF and plays an active role in mediating VEGF induced effects

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Abstract

Vascular endothelial growth factor (VEGF) and its receptor VEGFR-2 play important roles in mitogenesis and chemotaxis of endothelial cells. In normal human skin, VEGF is expressed and secreted by epidermal keratinocytes. Emerging data suggest that keratinocyte-derived VEGF targets other cell types besides the dermal endothelial cells. We have recently showed that keratinocytes from human normal skin expressed all five known VEGF receptors and co-receptors (neuropilin 1 and 2). To define the functional significance of VEGFR-2 in epidermis, we examined its role in a keratinocyte cell line, HaCaT cells, in response to VEGF treatment. Expression of VEGFR-2 on HaCaT cells was confirmed at both RNA and protein levels and was regulated by $VEGF_{165}$ treatment. Treatment of HaCaT cells with $VEGF_{165}$ induced tyrosine-autophosphorylation of VEGFR-2 and phosphorylation of PLC-γ and p44/42 MAPK in a time-dependent manner. Preincubation with a neutralizing antibody for VEGFR-2 (MAB3571) completely abrogated these phosphorylation effects. Furthermore, $VEGF_{165}$ stimulated proliferation and migration of HaCaT cells, and this effect was significantly blocked by a pretreatment with MAB3571. Neutralizing VEGFR-2 in HaCaT cells increased cell adhesion during culture. Our results suggest that VEGFR-2 expressed on HaCaT cells plays a crucial role in VEGF-mediated regulation of cell activity.

Keywords: VEGF; VEGFR-2; HaCaT cell; Keratinocyte; Phosphorylation; PLC-γ; p44/42 MAPK; Cell proliferation; Cell migration; Cell adhesion

Vascular endothelial growth factor (VEGF) is a dimeric glycoprotein and a hypoxia-inducible endothelial cell mitogen [1]. It acts as an essential regulator of vasculogenesis and haematopoiesis during embryonic development [2,3]. In adulthood, it drives angiogenesis in physiological conditions [2,3] and wound healing [4], as well as, in pathological conditions including cancer [5], rheumatoid arthritis [6], psoriasis [7], and cardiovascular disease [8]. VEGF increases vascular permeability [9] and stimulates endothelial cell migration and proliferation [10].

The effect of VEGF on endothelial cells is mainly mediated by two main receptor tyrosine kinases, VEGFR-1 (Flt-1) and VEGFR-2 (KDR or Flk-1) [1,2,11,12]. VEGFR-2 is a high-affinity receptor for VEGF [1,2,13]. It is tyrosine kinase phosphorylated much more efficiently upon ligand binding, leading to mitogenesis, chemotaxis, and morphological changes in endothelial cells [13,14]. VEGFR-2 is the primary transducer of VEGF signalling during physiological angiogenesis [1,2,13].

Although VEGF was originally thought to be highly specific for endothelial cells, it has become increasingly clear that it also elicits responses in non-endothelial cell types [15]. VEGF receptors have also been found to be expressed in certain non-endothelial cell types in the testis and epididymis, where overexpression of VEGF caused spermatogenic arrest, epithelial hyperplasia, and infertility [16]. In normal human skin, VEGF is expressed and secreted by epidermal keratinocytes [17]. Originally, the kerati-

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nocyte-derived VEGF was thought to act in a paracrine manner on vascular hyperpermeability and angiogenesis during wound healing [17]. Recently, others and our group have studied the expression of VEGF receptors (VEGFR-1, VEGFR-2, and VEGFR-3) and co-receptors (Neutropilin-1 and Neutropilin-2) in skin specimens and epidermal cells, but the results were not all consistent [18–21]. For instance, VEGFR-1, VEGFR-2, and NRP-1 were detected in normal human melanocytes [21]; VEGFR-1, but not VEGFR-2, was found to be expressed and played an active role in murine keratinocytes during wound repair and in normal human epidermal keratinocytes [20]. However, we found that keratinocytes in epidermis expressed all five known VEGF receptors and co-receptors [19]. Most importantly, neutralizing VEGFR-2 could block VEGF induced proliferation and migration of cultured normal human epidermal keratinocytes [19]. These findings promoted us to further investigate the potential role of VEGFR-2 in keratinocytes. In this report, we determined the expression of VEGFR-2 on a keratinocyte cell line, HaCaT cell, which is an HPV-negative immortalized keratinocyte cell line with mutant p53 alleles and stable expression of the mutant protein [22]. We showed evidence that VEGF regulated the expression and phosphorylation of VEGFR-2 in HaCaT cells. Blockage of VEGFR-2 by a neutralizing antibody could abrogate VEGF induced effects.

Materials and methods

Chemicals and reagents. High glucose DMEM, trypsin/trypsin–EDTA, fetal bovine serum, and Trizol reagent were obtained from Gibco and Invitrogen (Invitrogen, Auckland, USA). Monoclonal mouse anti-human VEGFR-2 antibodies were purchased from R&D Systems (MAB3571; Minneapolis, MN, USA) and Santa Cruz Biotechnology (SC-6251; Santa Cruz, CA, USA). Rabbit anti-human phospho-VEGFR-2 (Tyr1175), PLC-γ1, phospho-PLC-γ1 (Tyr783), p44/42 MAP kinase antibodies and mouse anti-human phospho-p44/42 MAPK (Thr202/Tyr204) antibody were obtained from Cell Signaling Technology (Beverly, MA, USA). Mouse anti-human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was from Acris Antibodies GmbH (Hiddenhausen, Germany). Horseradish peroxidase linked anti-mouse IgG was from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). Polyclonal rabbit anti-mouse immunoglobulins/FITC from DakoCytomation (Denmark A/S, Denmark). $VEGF_{165}$ was obtained from Chemicon International Inc. (Temecula, CA, USA). PVDF membrane, Whattman filter, and ECL plus reagent were purchased from Amersham Biosciences (Piscataway, NJ, USA). Moloney murine leukemia virus (MMLV) reverse transcriptase and RNase inhibitor were obtained from Fermantas (MBI Fermantas, Amherst, NY, USA). Cocktail protease inhibitor was purchased from Roche (Roche Diagnostics, Indianapolis, IN, USA). Propidium iodide (PI) was obtained from Sigma (Sigma–Aldrich, USA).

Cell culture. HaCaT cells were cultured in high glucose DMEM supplemented with 10% fetal bovine serum (FBS) and penicillin–streptomycin. The cells were maintained under standard cell culture conditions at 37 °C and 5% CO_2 in a humid environment. Human umbilical vein endothelial cells (HUVECs), serving as a positive control, were obtained from isolated umbilical veins by a standard method [23] and were cultured in human endothelial-SFM supplemented with 10% FBS.

Indirect immunofluorescence assay for VEGFR-2. HaCaT cells were seeded on coverslips in six-well culture plates at a density of 1×10^4 cells/ml. When the cells were 60–70% confluent, the coverslips were then fixed with 4% paraformaldehyde buffer for 20 min at room temperature. Next, coverslips were rinsed three times with PBS, transferred into preheated sodium citrate buffer (10 mM sodium citrate buffer, pH 8.5), and incubated at 95 °C for 20 min, then were rinsed three times in PBS. The cells were permeabilized at room temperature with PBS containing 0.1% Triton X-100 for 15 min and incubated with 10% normal rabbit serum for 1 h at room temperature to avoid non-specific binding. The coverslips were incubated with 20 μg/ml of primary monoclonal antibody against VEGFR-2 (MAB3571 or SC-6251) in PBS with 10% rabbit serum overnight at 4 °C, washed three times with PBS containing 1% rabbit serum, followed by incubation for 2 h at room temperature in the dark with FITC-conjugated rabbit anti-mouse secondary antibody that was diluted 1:40 with PBS containing 10% rabbit serum. After three washes with PBS containing 1% rabbit serum, the slides were counterstained with PI mounting medium to visualize the nuclei and analyzed by a fluorescence microscopy (Olympus, Japan). Images were captured with an attached Olympus digital camera. For each experiment, a negative control incubated with non-immune mouse IgG was included.

Reverse transcription and polymerase chain reaction (RT-PCR). HaCaT cells were grown to 80–90% confluent, then switched to DMEM supplemented with 0.5% BSA in the presence of various concentrations of $VEGF_{165}$ (0, 1, 5, 10, 25, 50, and 100 ng/ml) for 24 h. Total RNA was isolated from these cells by using Trizol reagent according to the manufacturer's instructions. Complementary DNA (cDNA) was subsequently synthesized from 2 μg of total RNA in a reaction volume of 20 μl, as described in our recent study [19]. Each experiment included samples containing no reverse transcriptase (negative controls) to exclude amplification from contaminating genomic DNA. RT-PCR for VEGFR-2 and GAPDH was performed using the same conditions and primer pairs (VEGFR-2: 5′-CTGGCGGCACGAAATATCCTCTTA-3′/5′-GGCCGGCTCTTTCGCTTACTGTTC-3′; *GAPDH*: 5′-TCATGTTTGAGACCTTCAA-3′/5′-GTCTTTGCGGATGTCCACG-3′) as described in [19]. In brief, 1 μl of cDNA was amplified in a volume of 50 μl that contained 10 pmol of each specific primer, 200 mM dNTP, 2 mM $MgCl_2$, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, and 2 units of *Taq* DNA polymerase. The thermal condition includes a pre-denaturation at 96 °C for 3 min, 35 cycles of 96 °C for 1 min, 57 °C for 1 min and 10 s, and 72 °C for 1 min and 20 s, and a full extension for 7 min at 72 °C. Results were expressed for each sample as band intensity relative to that of *GAPDH*.

Western blotting analysis. HaCaT cells were grown to 80–90% confluent and were harvested as before [19]. In brief, cells were washed twice in ice-cold PBS, scraped, and centrifuged (1000*g*, 5 min at 4 °C). The pellet was incubated for 30 min in modified RIPA lysis buffer (20 mM Tris–HCl [pH 7.5], 150 mM NaCl, 0.2 mM EDTA, 1% Triton X-114, 50 mM Hepes, 50 mM sodium fluoride, 1 mM sodium orthovanadate,1 tablet/10 ml of Protease Inhibitor Cocktail Tablets), then centrifuged (16,000*g*, 15 min at 4 °C). Protein concentration was measured using the QuantiPro BCA assay kit (Sigma–Aldrich, St. Louis, MO). Cell lysate was boiled in 2× Laemmli buffer (0.125 mol/L Tris–HCl, pH 6.8, 4% SDS, 20% glycerol, and 10% β-mercaptoethanol, 1% bromophenol blue) for 10 min and 40 μg of protein was loaded for each lane.

To determine the effect of $VEGF_{165}$ treatment on the VEGFR-2 expression, HaCaT cells (80–90% confluent) were washed twice in PBS and incubated in various concentrations of $VEGF_{165}$ (0, 1, 5, 10, 25, 50, and 100 ng/ml) in DMEM supplemented with 0.5% BSA for 24 h before harvest. Total cellular protein was separated by 6% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to PVDF membrane. Blots were blocked for 30 min at room temperature in PBS (pH 7.5) containing 1% Tween 20. After being rinsed three times for 10 min with PBS containing 0.05% Tween 20, the membrane was incubated with the primary antibody to VEGFR-2 (0.5 μg/ml) overnight at 4 °C in PBS containing 1% Tween 20. Blots were then washed four times (each 5 min) in PBS containing 0.05% Tween 20 and were incubated for 1 h with horseradish peroxidase-conjugated goat polyclonal anti-mouse IgG antibody (1:5000, Jackson). The membrane was washed five times (each 5 min) with 0.05% Tween 20 in PBS. The immunoreactive bands were detected by using enhanced chemiluminescent (ECL) plus reagent kit. The results of densitometric scanning of immunoreactivity on each lane were normalized by blotting for *GAPDH*.

For phosphorylation detection, nearly confluent HaCaT cells were serum starved for 24 h, then treated with 10 ng/ml $VEGF_{165}$ for 5, 15, and 30 min. In the blockade experiments, the serum-starved cells were pretreated with the VEGFR-2 neutralizing antibody, MAB3571 (5 μg/ml) for 30 min, followed by a treatment with 10 ng/ml $VEGF_{165}$ for 5 min. Cell lysate for phospho-VEGFR-2 was resolved in 6% SDS–PAGE, whereas for phospho-PLC-γ1, p44/42 MAPK, and phospho-p44/42 MAPK were resolved in 11% SDS–PAGE. Primary antibodies were diluted at 1:1000.

HaCaT cell proliferation assay. Proliferation assay of HaCaT cells was performed by MTT as described [19,24]. Briefly, HaCaT cells were trypsinized, counted, resuspended in DMEM with 10% FBS (5×10^4 cells/ml), and seeded into 96-well plates (100 μl/well). After 24 h, when cells were about 60–70% confluent, various concentrations of $VEGF_{165}$ (0, 1, 5, 10, 25, 50, and 100 ng/ml) were added in serum-free DMEM with 0.5% BSA for 24 h. For blockage assay, HaCaT cells were pre-incubated for 1 h with MAB3571 before adding VEGF. At the end of VEGF treatment, 20 μl of freshly prepared and filtered 3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide (MTT; Sigma, St. Louis, MO, USA) at a final concentration of 5 mg/ml in PBS was added into each well. The mixture was incubated for 3 h. Plates were flicked to remove medium and freeze–thawed twice. Cells were dissolved with 100 μl dimethylsulfoxide per well, and absorption was read at 570 nm with a spectrophotometric reader Elx800 (Bio-Tek Instruments, Winooski, VT, USA). All MTT assays were conducted in triplicate.

Cell migration assay. Cell migration assays was performed as previously described [25,26] with partial modification. Modified Boyden chambers (Neuroprobe, Bethesda, MD) with 8 μm porosity polyvinylpyrrolidone-free polycarbonate filters (Neuroprobe, Bethesda, MD) were precoated with 20 μg/ml type IV collagen (Sigma, USA). In the lower chamber, 27 μl various concentrations of VEGF in serum-free DMEM containing 0.5% BSA was added. The cell suspension (5×10^4/ml) was prepared and incubated with or without 5 μg/ml anti-human VEGFR-2 antibody MAB3571 for 30 min. Fifty microliters of cell suspension was added to the upper chamber, and cells were allowed to migrate for 8 h in culture condition. Then the upper surface of the membrane was wiped carefully to remove non-migratory cells. Cells that had migrated to the undersurface of the filter were fixed with 4% paraformaldehyde for 10 min and then stained with 2% crystal violet blue for 5 min. The filters were rinsed off thoroughly with running water. Migrating cells were quantified at 400× magnification in five randomly chosen fields per well. Each determination represents the mean of three individual wells ± SD. For all experiments, assays were repeated at least three times with similar results.

Cell adhesion assay. HaCaT cells were plated in 96-well plates at a concentration of 1×10^5 cells/well in serum-free DMEM containing 0.5% BSA and different concentrations of $VEGF_{165}$, and were incubated for 4 h at 37 °C in 5% CO_2. Cells were rinsed gently three times with 0.1% BSA in prewarmed culture medium to remove non-adherent cells. Adherent cells were then incubated with 5 mg/ml MTT for 3 h. The following procedures were similar to those in the proliferation assay. For VEGFR-2 blockage assay, cells pretreated with blocking antibody MAB3571 at 5 μg/ml for 30 min were incubated with or without VEGF (10 ng/ml) in the plates. The data was expressed as the mean of triplicate wells ± SD. Experiments were repeated at least in triplicate with similar results.

Statistical analyses. Results are expressed as means ± SD. All determinations were performed in triplicate, and experiments were repeated three times unless otherwise indicated. One-way ANOVA was used to evaluate significant differences. All statistical analyses were done by SPSS Software (V13.0, SPSS Inc. USA), with a P value < 0.05 considered to be statistically significant.

Results

HaCaT cells express VEGFR-2 mRNA and protein

The expression of VEGFR-2 in HaCaT cells was detectable by RT-PCR, Western blot, and indirect immunofluorescence assay (Fig. 1). Two monoclonal antibodies to VEGFR-2, MAB3571, and SC-6251, provided consistent results in Western blot and immunofluorescence assay. A representative immunoblotting with VEGFR-2 specific antibodies showed two bands at about 180 kDa and 200 kDa (indicating the glycosylation or phosphorylation of VEGFR-2) in both HUVECs and HaCaT cell lysates (Fig. 1C). Immunostaining for VEGFR-2 showed strong signal on the membrane of HaCaT cells (Fig. 1E).

VEGF165 regulates the expression of VEGFR-2 in HaCaT cells

In order to test whether VEGF affect the expression of VEGFR-2, HaCaT cells were incubated with varied concentrations of $VEGF_{165}$ in serum-free DMEM containing 0.5% BSA for 24 h. $VEGF_{165}$ maximally enhanced the expression of VEGFR-2 mRNA at a concentration of 10 ng/ml, while higher concentration of VEGF did not increase the stimulation effect (Fig. 2A). In parallel with mRNA expression, VEGFR-2 protein was also peaked at a VEGF concentration of 10 ng/ml (Fig. 2C).

VEGF165 induces phosphorylation of VEGFR-2, PLC-γ1, and p44/42 MAPK in HaCaT cells

VEGFR-2, which has intrinsic kinase activity, was tyrosine-phosphorylated in response to VEGF (10 ng/ml) treatment (Fig. 3A). Phosphorylation of VEGFR-2 reached peak level at 5 min and regressed to the baseline level at 30 min. Phosphorylation of PLC-γ1 was elevated by VEGF treatment and reached the maximum level at 15 min, then gradually went back to the basal level (Fig. 3B). Enhanced MAPK phosphorylation by $VEGF_{165}$ was found as early as 5 min and sustained at 15 min, but regressed to the baseline at 30 min (Fig. 3D). The above phosphorylation effects in response to VEGF treatment were completely blocked by a pre-incubation with VEGFR-2 neutralizing antibody (Fig. 3).

VEGFR-2 is involved in the proliferation, migration, and adhesion of HaCaT cells in response to VEGF treatment

$VEGF_{165}$ increased the proliferation of HaCaT cells in a dose-dependent manner (Fig. 4). VEGF at a concentration of 5 ng/ml could significantly increase the proliferating rate compared with untreated cells. VEGFR-2 neutralizing antibody, MAB3571, at a concentration of 5 μg/ml significantly inhibited proliferation of HaCaT cells: for cells pretreated with and without MAB3571 alone, P value = 0.047; for cells pretreated with and without MAB3571 in the presence of 10 ng/ml VEGF, the P value = 0.034 (Fig. 4).

The chemotactic motility of HaCaT cells was exponentially elevated by increasing $VEGF_{165}$ concentration (Fig. 5). The number of migrated cells at 100 ng/ml of $VEGF_{165}$ increased about 10-fold over the untreated. In the presence of 5 μg/ml VEGFR-2 neutralizing antibody

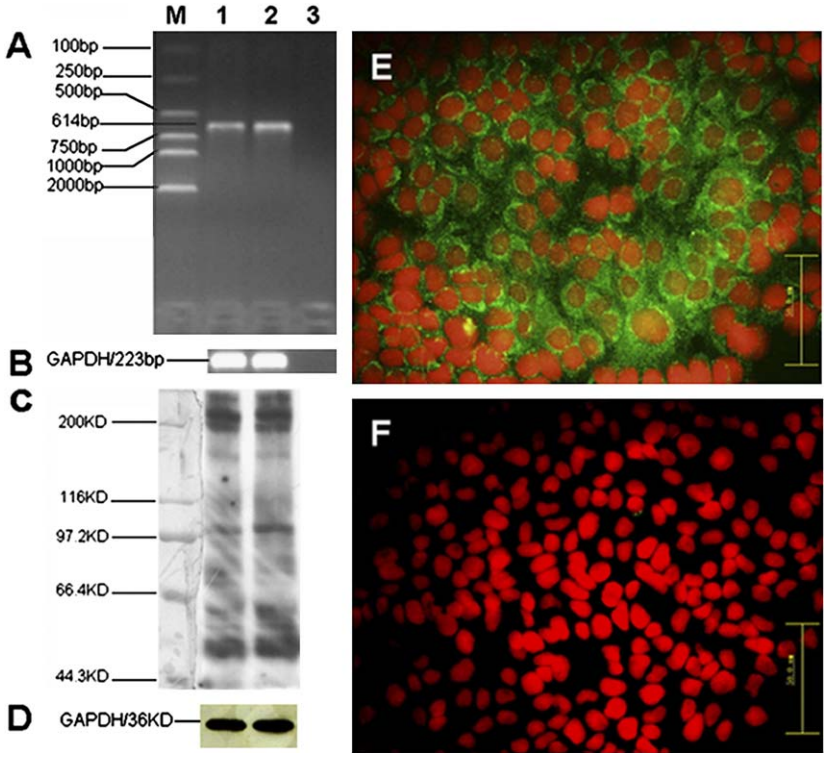

Fig. 1. Expression of VEGFR-2 on HaCaT cells. (A) RT-PCR analysis of VEGFR-2 mRNA in HaCaT cells. Lane M, DNA marker; lane 1, HUVEC as a positive control; lane 2, HaCaT cells; lane 3, negative control, in which the template was from samples containing no reverse transcriptase but all the other components during the reverse transcription. (B) GAPDH served as an internal control for mRNA. (C) Western blot for VEGFR-2 in protein extracted from exponential growth HaCaT cells (lane 2) and HUVECs (lane 1). (D) GAPDH served as a loading control for protein normalization. (E) Indirect immunofluorescence assay for VEGFR-2. Strongly immunostaining for VEGFR-2 (green color) was observed mainly on the cell membrane. (F) Staining with non-immune mouse IgG as a negative control. The cellular nuclei were stained red with PI. Scale bar: 50 μm.

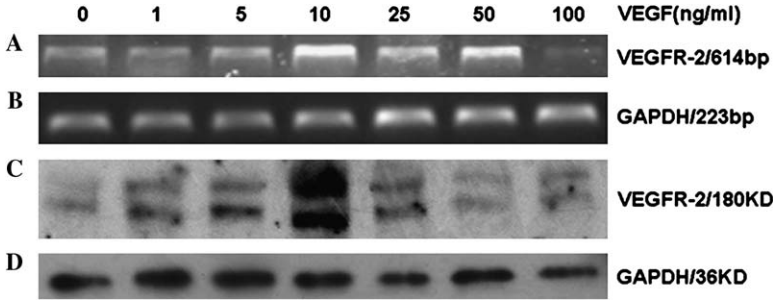

Fig. 2. Effect of $VEGF_{165}$ on mRNA and protein levels of VEGFR-2 in HaCaT cells. HaCaT cells were cultured to 80–90% confluent in growth medium, then switched to DMEM supplemented with 0.5% BSA and various concentrations of $VEGF_{165}$ (0, 1, 5, 10, 25, 50, and 100 ng/ml) for 24 h before harvest. (A) RT-PCR analysis of VEGFR-2 mRNA expression in HaCaT cells in response to different concentrations of $VEGF_{165}$. (B) GAPDH as internal control. (C) Western blotting analysis of VEGFR-2 in HaCaT cells exposed to $VEGF_{165}$. (D) GAPDH as a loading control for protein normalization.

and 10 ng/ml $VEGF_{165}$, the migration ability of HaCaT cells was reduced markedly by 2-fold compared with the $VEGF_{165}$ (10 ng/ml) treatment alone.

Under low concentrations of $VEGF_{165}$ (1–5 ng/ml) stimulation, the adhesion of HaCaT cells to culture plates was not significantly reduced compared with untreated cells. $VEGF_{165}$ decreased the adhesion of HaCaT cells by approximately 20% at the concentration of 10 ng/ml and the effect was more prominent by increasing the concentrations of $VEGF_{165}$ (Fig. 6). Pretreatment of HaCaT cells with MAB3571 alone significantly increased the amount of cells adhered to culture plates compared with untreated cells. This increase effect by VEGFR-2 neutralizing antibody was still remarkable even in the presence of $VEGF_{165}$ (10 ng/ml) (Fig. 6).

Discussion

Compelling evidence has suggested that VEGF and its receptors, especially VEGFR-2, play a critical role in angiogenesis under both physiological and pathological conditions [1–5,27,28]. Expression of VEGFR-2 has been found

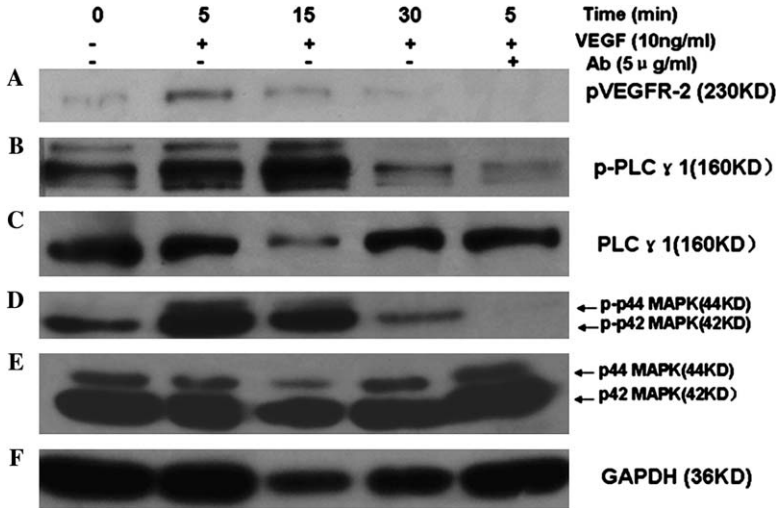

Fig. 3. $VEGF_{165}$ promotes phosphorylation of VEGFR-2, PLC-γ1, and p44/42 MAPK in HaCaT cells. Serum-starved HaCaT cells were either unstimulated or stimulated with $VEGF_{165}$ (10 ng/ml) for 5, 15 or 30 min. Cells pretreated with MAB3571 were also incubated with 10 ng/ml $VEGF_{165}$ for 5 min. Phosphorylation of VEGFR-2, PLC-γ1, and p44/42 MAPK in HaCaT cells responded to VEGF in a time-dependent manner. MAB3571 pretreatment completely blocked $VEGF_{165}$ induced phosphorylations. (A) Phospho-VEGFR-2. (B) Enhanced phosphorylation of PLC-γ1 by VEGF. (C) Total PLC-γ1. (D) Phospho-p44/42. No phospho-p44 MAPK bands were detected in untreated cells or cells treated with VEGF for 30 min. (E) Total p44 MAPK and p42 MAPK. (F) GAPDH, which served as loading control for protein normalization.

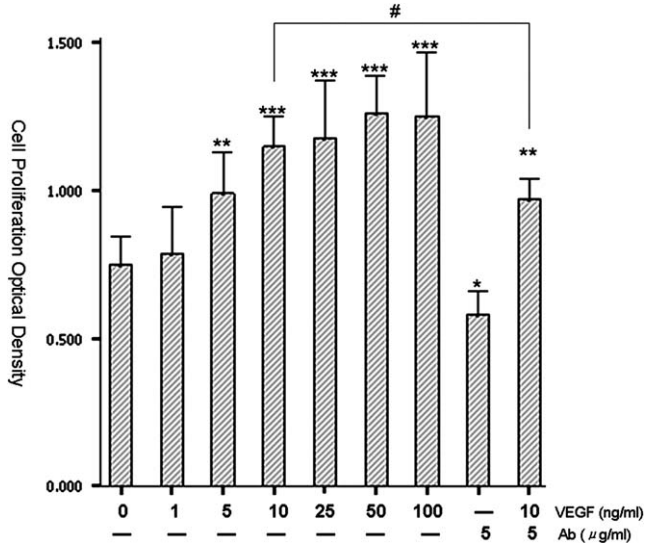

Fig. 4. $VEGF_{165}$ increases proliferation of HaCaT cells. MTT-based assay was performed to determine the effects of VEGF in the absence and presence of MAB3571. HaCaT cells were grown to 60–70% confluent in 96-well plates, switched into serum-free DMEM with 0.5% BSA, and treated with different concentrations of $VEGF_{165}$ (0, 1, 5, 10, 25, 50, and 100 ng/ml) for 24 h with and without MAB3571. VEGF significantly increased the proliferation of HaCaT cells at a concentration above 10 ng/ml. Data are expressed as values of optical density measured in six wells for each treatment. The experiment was repeated at least three times, with similar results. Adding VEGFR-2 antibody decreased cell proliferation induced by VEGF. The asterisks indicate significant differences ($^{*}P < 0.05$, $^{**}P \leqslant 0.01$, $^{***}P < 0.001$) between VEGF treated and untreated control; number sign show significant differences ($^{\#}P < 0.05$) between VEGF treated ± MAB3571.

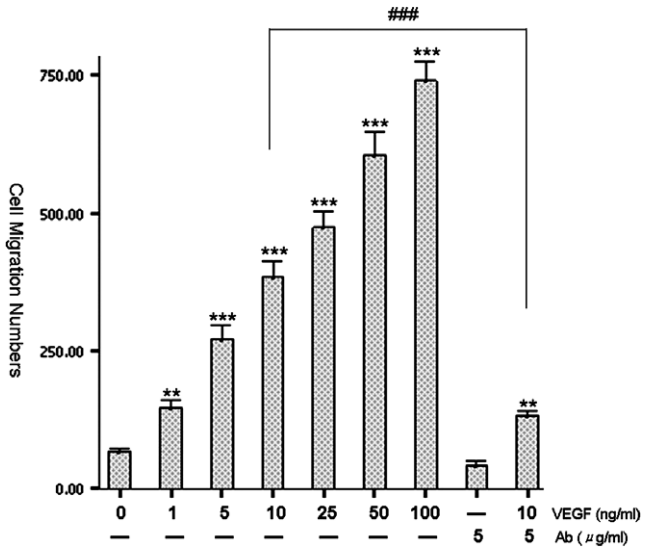

Fig. 5. $VEGF_{165}$ increases HaCaT cells migration. Data are expressed as the mean of three individual wells ± SD. Each well was counted for five randomly chosen fields at 400× magnification under microscope. The experiments were repeated at least three times, with similar results. The asterisks indicate significant differences ($^{**}P \leqslant 0.01$, $^{***}P < 0.001$) between VEGF treated and untreated control; number signs show significant differences ($^{\#\#\#}P < 0.001$) between VEGF treated cells ± MAB3571.

in some non-endothelial cell types, such as hematopoietic stem cells, megakaryocytes, neutrophils [29], monocytes [30], neuronal cells, pancreatic duct cells, and retinal progenitor cells [1,8], osteoblasts, stromal cells [1], and tumorigenic cell types [1,31]. In our recent study, we have found that keratinocytes in epidermis expressed all five known VEGR receptors and co-receptors [19]. It is conceivable that the expression of these receptors should have unique function during the development and maintenance of skin and wound healing. Indeed, VEGFR-1 has been reported to be involved in cutaneous wound repair [20]. Expression of neutropilin-1 in keratinocytes was also characterized

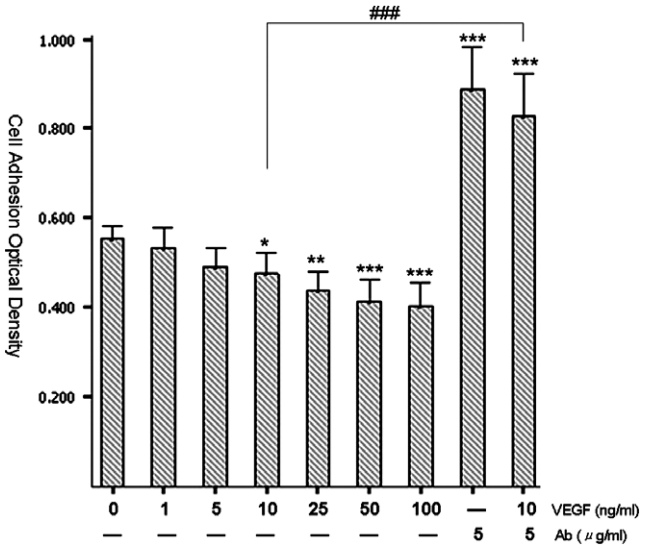

Fig. 6. $VEGF_{165}$ decreases HaCaT cell adhesion. HaCaT cells were plated in 96-well plates at a concentration of 1×10^5 cells/well in serum-free DMEM containing 0.5% BSA and different concentrations of $VEGF_{165}$, and were incubated for 4 h at 37 °C in 5% CO_2. After washing the cells, MTT-based assay was performed to quantify the number of attached cells. Optical absorbance values for VEGF treated HaCaT cells were lower than those for the untreated cells, which indicates a reduced rate of cell attachment. MAB3571 alone decreases cell adhesion compared with untreated cells. The asterisks indicate significant differences ($^{*}P < 0.05$, $^{**}P \leqslant 0.01$, $^{***}P < 0.001$) between VEGF treated and untreated control; number signs show significant differences ($^{\#\#\#}P < 0.001$) between VEGF treated cells ± MAB3571.

recently [18]. However, there are some conflicting results regarding the expression of VEGFRs in these studies: Wilgus et al. [20] reported that VEGFR-1, but not VEGFR-2, was detected in murine keratinocytes during wound repair and in normal human epidermal keratinocytes; Kurschat et al. [18] did not observe the expression of VEGFR-1 and VEGFR-2 in cultured HaCaT cells; whereas in our study for human skin section and keratinocytes, we found expression of VEGFR-1, VEGFR-2, and VEGFR-3 [19]. In the current study, we extended our observation in cultured HaCaT cells and tried to characterize the function of VEGFR-2. Consistent with our results on human skin samples [19], we found expression of VEGFR-2 mRNA and protein on HaCaT cells (Fig. 1A and C). Histological staining with specific anti-VEGFR-2 antibody MAB3571 showed an intense immunostaining on the cell membranes (Fig. 1C). This positive staining could be well reproduced by applying a different monoclonal antibody (SC-6251) for VEGFR-2 (data not shown).

Cultured epidermal keratinocytes expressed two major isoforms of VEGF, $VEGF_{121}$ and $VEGF_{165}$ [32]. We then utilized the predominant and most effective isoform, $VEGF_{165}$, as the stimulator in our study to characterize its effect on HaCaT cells. Our data demonstrated that exogenous $VEGF_{165}$ upregulated the mRNA and protein levels of VEGFR-2 in HaCaT cells (Fig. 2), which was coincident with other published report [33]. This upregulation effect was only observed at low concentrations of $VEGF_{165}$ and reached the highest level at 10 ng/ml. Further increase of VEGF concentration downregulated the expression of VEGFR-2 compared with the level at 10 ng/ml. This kind of feedback inhibition at high concentrations of VEGF is intriguing. Expression of VEGFR-2 has also been found to be autoregulated and was upregulated by VEGF in endometrial capillaries of ovariectomized mice [34]. The upregulation effect of VEGF on VEGFR-2 indicates that there stands an undefined complex autoregulation mechanism in HaCaT cells. We speculate that too much VEGF might induce the ligand-dependent endocytosis and degradation of VEGFR-2 [35].

Treatment of exogenous $VEGF_{165}$ on HaCaT cells induced a cascade of phosphorylation effects on VEGFR-2 and downstream signaling pathway targets (Fig. 3). VEGFR-2 phosphorylation effect was remarkable at 5 min in HaCaT cells after incubation with VEGF, thus confirming that VEGFR-2 on HaCaT cells is a functional signal transmitting receptor. In the absence of exogenous VEGF, HaCaT cells showed a baseline VEGFR-2 phosphorylation (Fig. 3A), which may be due to the autocrine effect of VEGF that was secreted by themselves. Neutralization of VEGFR-2 by an antibody diminished the phosphorylation of VEGFR-2 induced by VEGF. Based on the above data, it is evident that there exists a functional VEGF-VEGFR-2 autocrine loop in HaCaT cells.

As shown in Fig. 3B and D, VEGF induced significant activation of PLC-γ and p44/42 MAPK in HaCaT cells and this phosphorylation effects could be well blocked by a pretreatment with neutralizing antibody for VEGFR-2. All these results indicated that the phosphrylation effects in HaCaT cells were mediated by VEGFR-2. This observation is consistent with the results in vascular endothelial cells, in which autophosphorylation of the Tyr1175 residue on VEGFR-2 created a high affinity interaction site with PLC-γ and mediated cell proliferation signals via the MAP kinase pathway [36]. Briefly, VEGF binds to the extracellular domain of the VEGFR-2 on HaCaT cell membrane and induces autophosphorylation of VEGFR-2. PLC-γ binds to the region containing phosphotyrosines, and is tyrosine-phosphorylated. Activated PLC-γ stimulates the MAP kinase cascade in HaCaT cells and mediates its downstream function, such as regulation of cell growth and differentiation [36–40]. Our observations of cell proliferation and migration of HaCaT cells in response to VEGF stimulation (Figs. 4–6) are most likely resulted from these phosphorylation effects. All the induced effects enacted by VEGF treatment could be well blocked by neutralizing VEGFR-2. These observations are also similar to those in monocytes and endothelial cells [30,41,42]. The exact mechanism underlying these processes is still not well characterized. However, our data confirmed that, in HaCaT cells, cell proliferation and migration are (at least partly) mediated by VEGFR-2 via VEGF–VEGFR-2–PLC-γ–MAPK pathway.

Another intriguing finding in the present study is that stimulation of $VEGF_{165}$ could reduce the adhesion ability

of HaCaT cells. This observation is different from that in endothelial cells, where VEGF is believed to induce expression of the vascular endothelial adhesion molecular (VCAM) [43] and intercellular adhesion molecule (ICAM)-1 [43–45] and enhance the adhesion ability [43,46]. Furthermore, the ability of HaCaT cell adhesion to culture plate is greatly increased by a neutralization of VEGFR-2. Notably, pretreatment with anti-VEGFR-2 antibody alone could increase adhesion ability of HaCaT cells at least two folds compared with untreated cells. We suspect this might be from the effect that neutralization of VEGFR-2 could block the potential VEGF autocrine pathway during the culture.

In short, our data demonstrated that the expression of VEGFR-2 in HaCaT cells was regulated by exogenous $VEGF_{165}$. In HaCaT cells, VEGF functions, at least partially, through the VEGFR-2–PLC-γ–MAPK pathway, and enhances cell proliferation and chemotactic migration. The exact mechanism of increased ability of cell adhesion by neutralizing VEGFR-2 remains unknown. Our results suggest for an autocrine signaling loop in HaCaT cell line.

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