Des-γ-carboxyl prothrombin (DCP) is a well recognized tumor marker for hepatocellular carcinoma. Previously, we have demonstrated that DCP stimulates cell proliferation in hepatocellular carcinoma cell lines through Met-Janus kinase 1 signal transducer and activator of transcription 3 signaling pathway. In the present study, we demonstrated that DCP induces both cell proliferation and migration in human umbilical vein endothelial cells. DCP was found to bind with the kinase insert domain receptor (KDR), alternatively referred to as vascular endothelial growth factor receptor-2. Furthermore, DCP induced autophosphorylation of KDR and its downstream effector phospholipase C-γ and mitogen-activated protein kinase (MAPK). To support these results, we showed that DCP-induced cell proliferation and cell migration were inhibited by KDR short interfering RNA, KDR kinase inhibitor, or MAPK inhibitor. In conclusion, these results indicate that DCP is a novel type of vascular endothelial growth factor that possesses potent mitogenic and migratory activities.

Among liver malignancies, hepatocellular carcinoma (HCC) is the primary one and one of the most common abdominal cancers in the world (1). HCC is known as a highly malignant disease with a poor prognosis (2). Current treatments include surgical resection, liver transplantation, and local ablation therapy, which are effective only in localized tumors (3). There is no effective therapy for patients with advanced HCC. Therefore, there is an urgency to identify a therapy to control tumor progression of HCC. As HCC is generally a highly vascular tumor, the progression of HCC is greatly related to active neovascularization (4–7), which is the crucial step in tumor development as it helps in the nutrient and oxygen supply. Evidence of the vital role of tumor angiogenesis in the progression and metastasis of HCC has been identified (8). New strategies have been pursued to inhibit neovascularization or to destroy existing tumor vessels, including direct targeting of endothelial cells and indirect targeting by inhibiting the release of proangiogenic growth factors by cancer or stromal cells in current treatments for miscellaneous cancers other than HCC (9). However, there are limited data referring to the role of various angiogenic and angiostatic factors in HCC development (10–12).

Angiogenesis is regulated by a complex process of multiple, sequential, and interdependent steps (13) that are known to be regulated by some soluble angiogenic factors secreted from the tumor cells, vascular endothelial cells, and infiltrating cells. In the first step, vascular endothelial cells begin to migrate and accumulate in the region where the angiogenic factors are produced (14). Among the angiogenic factors, vascular endothelial growth factor (VEGF) plays a major role as it robustly stimulates proliferation, migration, and morphogenesis of vascular endothelial cells (15–19). It has been reported that VEGF expression is up-regulated in HCC (20) and that VEGF expression is correlated with tumor angiogenesis of HCC (4). Others reported that serum VEGF can be a prognostic marker for HCC (21). However, Van Belle et al. (22) showed that when submaximal concentrations of VEGF and hepatocyte growth factor are used individually, there is no significant effect on endothelial cell proliferation and migration. This result implies that other growth factors may be involved in HCC-related angiogenesis.

Autocrine signaling could be another mechanism that contributes to the tumor progression of HCC. We previously reported that des-γ-carboxyl prothrombin (DCP) is an autologous growth factor for HCC (23). DCP is a well recognized tumor marker that is a prothrombin (PT) precursor with no coagulation activity. The prothrombin precursor has 10 glutamic acid (Glu) residues in the N terminus that are converted into γ-carboxy-glutamic acid (Gla) residues by vitamin K-dependent γ-glutamyl carboxylase. All of these Glu residues need to be converted into Gla residues before PT can obtain coagulation activity. In DCP, not all of the 10 Gla residues are transformed; some remain as Glu residues (24).

Many studies consider DCP as a prognostic indicator of HCC (25–30). Moreover, tumor arterial vascularity has indicated a strong correlation with the expression of both serum and tissue DCP (31). Our previous study demonstrated that DCP stimulates cell proliferation of the HCC cell line and that Met-Janus kinase (JAK) 1 signal transducer and activator of transcription (STAT) 3 signaling pathway is considered to be a major signaling pathway for its biological effect. As DCP is secreted from...
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HCC cells, DCP might work as a paracrine interaction factor between HCC cells and vascular endothelial cells. Hence, we assessed the effect of DCP on human umbilical vein endothelial cell (HUVEC) proliferation and migration as part of the process of angiogenesis. The transductional apparatus of DCP was identified to activate the phospholipase C-γ (PLC-γ) MAPK signaling pathway via activation of the kinase insert domain receptor (KDR), also known as vascular endothelial growth factor receptor 2. These findings provide a description of a novel paracrine mechanism that is involved in HCC development.

EXPERIMENTAL PROCEDURES

Cell Culture—HUVEC was obtained from Sanko Junyaku Co. Ltd. (Tokyo, Japan) and cultured in endothelial basal medium 2 (EGM™-2) (Sanko Junyaku) supplemented with SingleQuo™ containing 2% heat-inactivated fetal bovine serum, hydrocortisone, human basic fibroblast growth factor, VEGF, R3-insulin-like growth factor 1, ascorbic acid, heparin, human epidermal growth factor, gentamicin, and amphotericin-B (Sanko Junyaku). DCP was purified from the DCP-producing HCC cell line PLC/PRF/5 as described previously (23). Cells were cultured at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air. Cells were quiesced at subconfluence in serum-free conditions for 24 h before the experiment.

Cell Proliferation Assay—DNA synthesis was estimated by measuring [³H]thymidine incorporation. Cells were grown in 12-well tissue culture plastic dishes. After 24 h of quiescence, cells were treated with DCP, PT (Sigma), VEGF165 (PeproTech, Rocky Hill, NJ), if required, at the indicated concentrations for 18 h. [³H]thymidine (5 µCi/ml) (Amersham Biosciences) was added during the last 6 h of the 18-h treatment period. Cells were treated with 5% trichloroacetic acid for 30 min at 4 °C and harvested, and radioactivity was measured in a liquid scintillation counter (Beckman Coulter, Fullerton, CA).

Cell Migration Assay—Cells were plated into 6-well tissue culture plastic dishes. After 24 h of quiescence, experimental wounds were made by dragging a rubber policeman™ (Fisher Scientific, Hampton, NH) across the cell culture. The cultures were rinsed with phosphate-buffered saline and lysed in 150 µl of sample buffer (100 mM Tris-HCl, pH 6.8, 10% glycerol, 4% SDS, 1% bromphenol blue, 10% β-mercaptoethanol). The samples were resolved using SDS-PAGE and transferred to Immobilon-P™ polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA). The membranes were incubated with anti-phospho-Met (Tyr-1234/1235) (Cell Signaling Technology, Beverly, MA), anti-phospho-Met (Tyr-1349) (Cell Signaling Technology), anti-phospho-JAK 1 (Tyr-1022/1023) (Sigma), anti-phospho-STAT 3 (Tyr-704) (Sigma), anti-phospho-p44/42 MAPK (Thr-202/204) (Cell Signaling Technology), and anti-phospho-PLC-γ-1 (Tyr-771) (Upstate Cell Signaling, Lake Placid, NY) antibodies overnight at 4 °C. The membranes were washed three times in Tris-buffered saline with Tween 20 (TBS-T) buffer (Sigma), probed with horseradish peroxidase-conjugated secondary antibody, and developed with an ECL Western blotting detection system (Amersham Biosciences) using enhanced chemiluminescence.

Antibody Array Analysis—Cells were grown to confluence in 10-cm tissue culture plastic dishes, quiesced for 24 h, and stimulated with DCP (20 ng/ml), PT (20 ng/ml), or VEGF (10.6 ng/ml), if required, for 15 min. Human phospho-receptor tyrosine kinase array kit (Proteome Profiler™ Array, R&D Systems, Minneapolis, MN) was validated by profiling the tyrosine phosphorylation in HUVEC treated with DCP, PT, and VEGF. Conditioning of the membranes and hybridization were performed according to the manufacturer’s protocol. The signal was detected and quantified using a Lumimager F1 (Roche Applied Science).

Immunoprecipitation—Cells were plated and grown to confluence in 10-cm tissue culture plastic dishes and treated with DCP, PT, and VEGF, if required, at the indicated concentrations for 15 min at 37 °C. Cell lysates containing the same amount of proteins were immunoprecipitated with anti-KDR antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Immunocomplexes were captured with protein G-agarose beads (Santa Cruz Biotechnology), washed four times with 20 nM HEPES buffer, pH 7.4, containing 10% glycerol, 0.1% Triton X-100, 500 mM sodium chloride, 1 mM sodium vanadate. Precipitates were analyzed with 7.5% SDS-PAGE and immunoblotted with anti-DCP antibody (Eisai Co. Ltd., Tokyo, Japan) and anti-phospho-tyrosine antibody (R&D Systems).

Inhibitors of KDR-PLC-γ-MAPK Signaling Pathway—Function-blocking KDR kinase inhibitor V ZM323881 (Calbiochem) and MAPK kinase (MEK) inhibitor PD98059 (Biomol, Plymouth Meeting, PA) were utilized for the function-blocking assay against KDR-PLC-γ-MAPK signaling. The cells were treated with function-blocking ZM323881 (2 nM) or PD98059 (4 µM) with DCP stimulation.

Gene Silencing with Small Interfering RNA (siRNA)—siRNA duplexes targeting KDR sequences were obtained from Dharmacon SMART pool technology™ (Dharmacon Research, Lafayette, CO). Control (lamin A/C) siRNA duplex was obtained from Dharmacon. siRNAs were transfected into cells using RNAiFect™ transfection reagent (Qiagen). Cells were incubated for 12 h before the analysis. The interferences of KDR protein expression were confirmed by immunoblot analysis using anti-KDR antibody (Cell Signaling Technology) (data not shown).
FIGURE 1. Effect of DCP on DNA synthesis and cell migration in HUVEC. A, cells were treated with DCP, PT, or VEGF at the indicated concentrations for 18 h in cell proliferation assay. B, cells were treated with DCP, PT, or VEGF at the indicated concentrations for 24 h in cell migration assay. Cell proliferation activity and migrative activity were measured as described under "Experimental Procedures." The data are shown as the ratio to untreated control cells. The data are the mean ± S.E. of more than three independent studies (*, *p < 0.05; **, *p < 0.01; n.s., not significant (versus non-treatment); Student's t test). C, morphology of DCP-treated cells. Cells were plated in 6-well tissue culture plastic dishes with quiescent medium at the concentration of 10⁵ cells/ml. After 24 h of incubation, cells were treated with DCP (20 ng/ml), PT (20 ng/ml), or VEGF (10.6 ng/ml) for another 24 h, after which pictures were taken by phase-contrast microscopy. Pictures were representative fields from three independent experiments. Bar, 100 μm.
RESULTS

DCP Stimulates Cell Proliferation in HUVEC—To investigate the mitogenic effect of DCP, we utilized the incorporation of \[^{3}H\]thymidine into DNA as the index of proliferation. As shown in Fig. 1A, an increasing concentration of DCP led to increased incorporation of \[^{3}H\]thymidine in HUVEC. DCP stimulated cell proliferation by 1.48 ± 0.12-fold at a concentration of 20 ng/ml in HUVEC compared with the 1.10 ± 0.09-fold in normal PT at the same concentration. This effect reached a plateau at concentrations higher than 20 ng/ml (data not shown).

DCP Stimulates Cell Migration in HUVEC—DCP-induced cell migratory activities were assessed by in vitro wound healing assay. DCP-stimulated cell migration was 2.16 ± 0.29-fold at a concentration of 20 ng/ml in HUVEC compared with the 1.11 ± 0.25-fold in normal PT at the same concentration. As shown in Fig. 1B, an increasing concentration of DCP led to a significant promotion in the migrative activity in HUVEC. This effect also reached a plateau at the DCP stimulus of 20 ng/ml (data not shown).

DCP Does Not Induce Morphological Change in HUVEC—Cultured HUVEC was relatively heterogeneous in terms of morphology. DCP, PT, and VEGF induced no significant morphological change at 12 (data not shown) and 24 h (Fig. 1C) in HUVEC.

The Met-JAK-STAT Signaling Pathway Is Not Affected by DCP Stimulation—In previous studies of HCC cell lines, DCP was found to bind with the cell surface receptor Met and cause autophosphorylation (23). Activation of the Met-JAK-STAT signaling pathway was assessed in HUVEC, and DCP did not significantly induce phosphorylation of Met, JAK 1, or STAT 3 at 5 min (data not shown), 15 min, and 6 h (Fig. 2).

Biological Effect of DCP on Binding to KDR and Activation of KDR-PLC-γ-MAPK Signaling Pathway—Because DCP did not stimulate the Met-JAK-STAT signaling pathway, the activation of other receptor tyrosine kinases was screened using a human phospho-receptor tyrosine kinase array kit. The antibody array contains antibodies for phosphorylated receptor tyrosine kinases and related signaling molecules including Met, VEGF receptor, epidermal growth factor receptor, fibroblast growth factor receptor, platelet-derived growth factor receptor, insulin receptor, Axl, RET, retinoid-related orphan receptor, tyrosine kinase with immunoglobulin and epidermal growth factor homology domains, nerve growth factor receptor, muscle-specific receptor, and ephrin receptor. Among these receptors, phosphorylation of KDR was induced by DCP stimulation in HUVEC, whereas PT did not induce phosphorylation of KDR (Fig. 3). Met stimulation was not detected in the antibody array analysis, which was consistent with the results of the immunoblot analysis. Consequently, the direct binding between DCP and KDR was examined by immunoprecipitation assay. DCP was detected in the immunoprecipitant with anti-KDR antibody (Fig. 4A). This confirmed the antibody array analysis result, as detected by anti-phospho-tyrosine antibody in HUVEC, demonstrating DCP treatment induced autophosphorylation of KDR (Fig. 4B). When KDR is activated by its ligand VEGF, the specific tyrosine residues are reported to be autophosphorylated (32–34). It has been reported that the downstream pathway binding with VEGF-A and activation of KDR induce the binding of PLC-γ to KDR via its carboxy-terminal SH2 domain, as well as produce strong phosphorylation at tyrosine (34). Consequently the activated PLC-γ efficiently stimulates protein kinase C, resulting in the stimulation of the ras-MEK-MAPK pathway (34). To identify the signaling pathway following the autophosphorylation of KDR, activations of PLC-γ and MAPK were examined by immunoblot analysis of HUVEC treated with DCP. Phosphorylation of PLC-γ and MAPK was induced by DCP and KDR, but not PT (Fig. 4, C and D).
Abrogation of the DCP-induced Cell Proliferative and Cell Migrative Effect of DCP by KDR Inhibitor, MEK Inhibitor, and KDR siRNA—To determine whether the biological effect of DCP occurs through the KDR-PLC-γ-MAPK signaling pathway, HUVEC was treated with function-blocking KDR kinase inhibitor or MEK inhibitor. Cell proliferative activities were analyzed by [3H]thymidine incorporation assay. The inhibition of DCP-induced proliferative activity by KDR kinase inhibitor or MEK inhibitor reached 93 ± 14 and 79 ± 14%, respectively (Fig. 5A). VEGF-induced cell proliferation was partially inhibited by MEK inhibitor, suggesting that VEGF may work through other signaling pathway. Migrative activities were analyzed using an in vitro wound healing assay in HUVEC treated with KDR kinase inhibitor or MEK inhibitor. The inhibition of DCP-induced migrative activity by KDR kinase inhibitor or MEK inhibitor reached 104 ± 2 and 102 ± 12%, respectively (Fig. 5B). HUVEC was treated with KDR siRNA in [3H]thymidine incorporation assay or in vitro wound healing assay to determine whether the biological effect of DCP is due to the action of KDR. The inhibition of [3H]thymidine incorporation by KDR siRNA reached 115 ± 17% in HUVEC (Fig. 6A). The inhibition of cell migrative activity by KDR siRNA was 110 ± 24% in HUVEC (Fig. 6B).

**DISCUSSION**

In the current study, we present DCP as a potent paracrine stimulant for vascular endothelial cells. Clinical studies show a significant correlation between serum DCP level and the clinical angiogenic potential or vascularity of HCC (31). However, the role of DCP in angiogenesis of HCC has not been confirmed. In this study, we demonstrated that DCP directly stimulated cell proliferation (Fig. 1A) and cell migration (Fig. 1B) of vascular endothelial cells.

Previously, we reported that DCP acts as an autologous mitogen for HCC cell lines and that the biological effect of DCP is brought out through the Met-JAK-STAT signaling pathway (23). From that point of view, we assessed the DCP-induced activation of the Met-JAK-STAT signaling pathway in HUVEC. However, we found that DCP did not induce phosphorylation of Met, JAK, or STAT significantly (Fig. 2). It has been reported that Met is hardly detectable in quiescent confluent endothelial cells and differentiated endothelial cells on three-dimensional collagen gel (35). Besides, the process of angiogenesis is very complex and involves a number of orchestrated steps (13). We did not address the synergic effect of two receptors in this report. It is reported that semaphorin, Sema4D, is angiogenic and that its biological effect is mediated by its high affinity receptor, plexin B1 (36, 37). The biological effects elicited by plexin B1 require coupling and activation of the Met tyrosine kinase (37). Moreover, semaphorin induces a lower level of Met

![DCP-stimulated Vascular Endothelial Cells](image-url)
DCP-stimulated Vascular Endothelial Cells

![Diagram](Image)

**A**

| KDR siRNA | DCP | VEGF |
|-----------|-----|------|
| n.t.      |     |      |
| +         |     |      |

**B**

| KDR siRNA | DCP | VEGF |
|-----------|-----|------|
| n.t.      |     |      |
| +         |     |      |

**FIGURE 6. The effect of gene silencing using KDR siRNA on cell proliferation (A) and cell migration (B).** After treatment with KDR siRNA duplexes, cell proliferation and cell migration assays were performed as described under “Experimental Procedures.” The data are shown as the ratio to untreated control cells. The data are the mean ± S.E. of more than three independent studies. *, p < 0.05; **, p < 0.01; n.s., not significant (versus non-treatment of KDR siRNA; Student’s t test). n.t., nontreated control.

stimulation without other Met ligands in HUVEC (36). These findings imply that an undetectable level of Met activation may be involved in the biological effect induced by DCP stimulation. Further studies are necessary to probe the synergic effect of two receptors to clearly exclude the involvement of Met signaling.

Despite the fact that DCP stimulates HCC cells through the Met-JAK-STAT signaling pathway, it also stimulated an alternative cell surface receptor in HUVEC. Receptor antibody array analysis showed that DCP hit the cell surface receptor KDR in HUVEC. To the best of our knowledge, this is the first study that clearly demonstrates a stimulus effect on endothelial proliferation and migration.

HCC is characterized by high vascularity and arterial blood supply, rather than the portal blood supply, which increase as HCC develops (38). VEGF, a strong endothelial cell-specific mitogen, is known to be involved in angiogenesis of malignancies including HCC (17, 20, 39–42). Some studies indicated an overexpression of VEGF in HCC tissues compared with adjacent noncancerous liver tissue (20, 43, 44). However, others reported that VEGF expression is higher in the surrounding cirrhotic liver tissues than in the HCC. They also reported that basic fibroblast growth factor expression is correlated with VEGF expression (45). It has been suggested that VEGF expression may initiate angiogenesis in HCC tissue, although this view is still regarded as controversial.

It was hypothesized that VEGF secretion in HUVEC was stimulated by DCP because the KDR-PLC-γ-MAPK signaling pathway is activated by DCP as well as VEGF. VEGF consists of several subtypes, including VEGF-A, -B, -C, and -D. All members of the VEGF family have in common the ability to stimulate endothelial cell proliferation, but may act through different receptors: Fms-related tyrosine kinase receptor 1 (FLT1/VEGFR1) binds VEGF-A and -B (46, 47); KDR binds VEGF-A, -C, and -D (15, 48–50); Fms-like tyrosine kinase 4 (FLT4/VEGFR3) binds VEGF-C and -D (49, 50). As a result, endogenous VEGF-A, -C, and -D secretion should cause activation of not only KDR but also FLT1 or FLT4. The phosphorylations of FLT1 or FLT4 were not induced by DCP stimulation in antibody array analysis, whereas VEGF stimulation induced phosphorylation of FLT1 and KDR (Fig. 3). Enzyme-linked immunosorbent assay analysis showed that endogenous secretions of VEGF-A, -C, and -D were not induced by DCP stimulation in HUVEC (data not shown). Moreover, DCP induced autophosphorylation of KDR in 15 min. Endogenous VEGF secretion is too fast to involve KDR activation, as autophosphorylation of KDR was detected within 5 min of DCP exposure (data not shown). These results imply that DCP binds directly to KDR and activates KDR signaling.

As the best known ligand for the KDR is VEGF (51–54), we utilized VEGF165 as a positive control (reacted ligand). VEGF administered to HUVEC in the dose of 10.4 ng/ml, equivalent to 277 pm, induced both cell proliferation and migration (Fig. 1, A and B). This concentration is physiologically relevant, because the known dissociation constant of the VEGF-KDR complex is 41–770 pm (55–57). The concentration of DCP used in the current study is 20 ng/ml, which is equivalent to 278 pm. Thus, DCP displayed an activity similar to VEGF in terms of molar concentration. These findings also support our hypothesis that stimulation of KDR is elicited by direct binding of DCP. The binding of KDR to DCP was detected by co-immunoprecipitation analysis (Fig. 4A). It is reasonable to suppose that KDR-PLC-γ-MAPK signaling pathway is the major signaling pathway for DCP (Figs. 4, B–D, 5, and 6).

LeCouter and others (58–60) have reported that FLT1 and KDR signaling achieves multiple functions by activating both of these receptors, signaling selectively depending on cell type. In the current study, we utilized HUVEC, raising a question that hepatic sinusoidal endothelial cells might signal differently from HUVEC in response to VEGF. In the study of hepatic sinusoidal endothelial cells, KDR signaling is well preserved (58). This finding strongly supports our hypothesis that DCP stimulates vascular endothelial cell migration and proliferation in HCC development. Further study is necessary to elucidate the physiological and patho-physiological function of DCP on angiogenesis.
DCP produced the biological effect through the alternative cell surface receptor KDR in HUVEC. This might be a curious phenomenon as one ligand binds to a different cell surface receptor. There are some other cases where one ligand utilizes different receptors depending on the conditions. Bag-1 was demonstrated a novel paracrine mechanism of DCP, where the DCP-KDR-PLC-γ-MAPK signaling cascade linked to mitogenesis (61).

To the best of our knowledge, this is the first study that demonstrated a novel paracrine mechanism of DCP in vitro, where the DCP-KDR-PLC-γ-MAPK signaling pathway becomes a capable therapeutic target to inhibit the development of HCC with respect to angiogenesis. Further in vivo studies are necessary to validate this mechanism.

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REFERENCES

1. Okuda, K. (2000) J. Hepatol. 32, Suppl. 1, 225–237
2. Mise, K., Tashiro, S., Yogita, S., Wada, D., Harada, M., Fukuda, Y., Miyake, H., Isikawa, M., Izumi, K., and Sano, N. (1998) Clin. Cancer Res. 4, 1475–1482
3. Mulcahy, M. F. (2005) Curr. Treat. Options Oncol. 6, 423–435
4. Park, Y. N., Kim, Y. B., Yang, K. M., and Park, C. (2000) Arch. Pathol. Lab. Med. 124, 1061–1065
5. Terada, T., and Nakanuma, Y. (1995) Histopathology 27, 333–339
6. Roncalli, M., Roz, E., Coggi, G., Di Rocco, M. G., Bossi, P., Minola, E., Gambacorta, M., and Bonzio, M. (1999) Hepatology 30, 1174–1178
7. Poorn, R. T., Ng, I. O., Lau, C., Zhu, L. X., Yu, W. C., Lo, C. M., Fan, S. T., and Wong, J. (2001) Ann. Surg. 233, 227–235
8. Sugimachi, K., Tanaka, S., Terashi, T., Taguchi, K., and Rickituri, T. (2002) Surgery 131, Suppl. 1, S135-S141
9. Jain, R. K., Duda, D. G., Clark, I. W., and Loeffer, J. S. (2006) Nat. Clin. Pract. Oncol. 3, 24–40
10. Miura, H., Miyazaki, T., Kuroda, M., Oka, T., Machinami, R., Kodama, T., Harada, M., Fukuda, Y., Miyake, H., Isikawa, M., Izumi, K., and Sano, N. (1998) Clin. Cancer Res. 4, 1475–1482

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Pelletier, N., and Ferrara, N. (2001) *J. Biol. Chem.*, **276**, 3222–3230

55. Waltenberger, J., Claesson-Welsh, L., Siegbahn, A., Shibuya, M., and Heldin, C. H. (1994) *J. Biol. Chem.*, **269**, 26988–26995

56. Fuh, G., Li, B., Crowley, C., Cunningham, B., and Wells, J. A. (1998) *J. Biol. Chem.*, **273**, 11197–11204

57. Li, B., Ogasawara, A. K., Yang, R., Wei, W., He, G. W., Zioncheck, T. F., Bunting, S., de Vos, A. M., and Jin, H. (2002) *Hypertension* **39**, 1095–1100

58. LeCouter, J., Moritz, D. R., Li, B., Phillips, G. L., Liang, X. H., Gerber, H. P., Hillan, K. J., and Ferrara, N. (2003) *Science* **299**, 890–893

59. Park, J. E., Chen, H. H., Winer, J., Houck, K. A., and Ferrara, N. (1994) *J. Biol. Chem.*, **269**, 25646–25654

60. Zeng, H., Dvorak, H. F., and Mukhopadhyay, D. (2001) *J. Biol. Chem.*, **276**, 26969–26979

61. Bardelli, A., Longati, P., Albero, D., Goruppi, S., Schneider, C., Ponzetto, C., and Comoglio, P. M. (1996) *EMBO J.* **15**, 6205–6212