Kisspeptin-10, a KISS1-Derived Decapeptide, Inhibits Tumor Angiogenesis by Suppressing Sp1-Mediated VEGF Expression and FAK/Rho GTPase Activation

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Abstract

Kisspeptin-10 (Kp-10), a decapeptide derived from the primary translation product of KISS1 gene, has been reported previously to be a key hormone for puberty and an inhibitor for tumor metastasis via the activation of G protein–coupled receptor 54. However, whether Kp-10 inhibits angiogenesis, which is critical for tumor growth and metastasis and other human diseases, is still unknown. Here we show that Kp-10 significantly inhibits human umbilical vein endothelial cell (HUVEC) migration, invasion, and tube formation, key processes in angiogenesis. Using chicken chorioallantoic membrane assay and vascular endothelial growth factor (VEGF)–induced mouse corneal micropocket assay, we show that Kp-10 inhibits angiogenesis in vivo. Furthermore, Kp-10 inhibits tumor growth in severe combined immunodeficient mouse xenografted with human prostate cancer cells (PC-3) through inhibiting tumor angiogenesis, whereas Kp-10 has little effect on the proliferation of HUVECs and human prostate cancer cells. In deciphering the underlying molecular mechanisms, we show that Kp-10 suppresses VEGF expression by inhibiting the binding of specificity protein 1 to VEGF promoter and by blocking the activation of c-Src/focal adhesion kinase and Rac/Cdc42 signaling pathways in HUVECs, leading to the inhibition of tumor angiogenesis. [Cancer Res 2009;69(17):OF1–9]

Introduction

Angiogenesis, the growth of new blood vessels from preexisting vessels, is crucial for tumor growth and metastasis (1). Numerous previous reports showed that it is effective to inhibit tumor growth and metastasis by blocking tumor angiogenesis (2). Angiogenesis is a complex process including endothelial cell proliferation, migration, and tube formation. Vascular endothelial growth factor (VEGF), secreted from tumor cells and endothelial cells through either autocrine or paracrine manner, is critical for all steps of tumor progression, including tumor cell growth and angiogenesis (3–5). VEGF expression is regulated by either hypoxia-dependent or hypoxia-independent pathways. The high GC-rich motifs in the proximal regions of VEGF promoter are regulated by transcriptional factor specificity protein 1 (Sp1; refs. 6, 7), suggesting that Sp1 is implicated for the basal VEGF expression level in cells (8–11). When VEGF secreted by tumor cells interacts with VEGF receptor-2 in endothelial cells, VEGF receptor-2–binding c-Src recruits and fully activates focal adhesion kinase (FAK), which in turn stimulates downstream proteins such as Rho GTPases or mitogen-activated protein kinases. Therefore, a decrease in VEGF expression or inhibition of VEGF-mediated signaling pathways in endothelial cells is important for the inhibition of tumor angiogenesis (12).

Human KISS1 gene encodes a premature 145–amino acid protein, which is proteolytically cleaved into polypeptides known as kisspeptins, including Kp-54 (also called metastin), Kp-14, Kp-13, and kisspeptin-10 (Kp-10; refs. 13–15). Kisspeptins were originally identified as a metastasis suppressor in melanoma cells via binding and activating their cognate receptor G protein–coupled receptor 54 (GPR54; refs. 14, 16–18). Further studies showed that loss of KISS1 gene expression was correlated with increased metastasis and/or tumor progression in a wide variety of tumor types, including malignant pheochromocytoma, esophageal squamous cell carcinoma, bladder tumor, ovarian, gastric, and pancreatic tumors (19–29). On the other hand, an increase in KISS1 and GPR54 expression with high-grade and metastatic capacity has been reported in breast tumors and hepatocellular carcinomas (30–32). Recently, increased interests of kisspeptins focused on their key roles in the regulation of the hypothalamic-pituitary-gonadal axis during puberty and reproductive development (11, 33–38). Among different kisspeptins, Kp-10 is the shortest peptide and highly conserved between mouse and human with one conserved amino acid replacement (18). Previous studies reported that Kp-10 regulated pubertal signaling in hypothalamus (38, 39), inhibited cell migration of primary human trophoblasts in placenta, suppressed metastatic activity of tumor cells (40), and acted as a vasoconstrctor (41). However, it is still unclear whether human Kp-10 inhibits angiogenesis or tumor angiogenesis. In this study, we investigated the effects of human Kp-10 on tumor angiogenesis and the underlying molecular mechanisms. We found that Kp-10 inhibited endothelial cell migration, invasion, and tube formation. Using chicken embryo chorioallantoic membrane (CAM) assay and mouse corneal micropocket models, we show that Kp-10 inhibits angiogenesis in vivo. Furthermore, Kp-10 inhibited tumor growth by suppressing tumor angiogenesis in xenograft mouse models with human prostate cancer cells. To understand the molecular mechanism of Kp-10 in regulating angiogenesis, we showed that Kp-10 inhibited Sp1-mediated VEGF expression in tumor cells and suppressed the endothelial cell
migration by blocking the activation of c-Src/FAK and Rac/Cdc42 GTPase. Therefore, our data suggest that Kp-10 inhibits angiogenesis and tumor growth by blocking Sp1-mediated VEGF expression and suppressing c-Src/FAK signaling pathway activation.

Materials and Methods

Cells, cell culture, reagents, and animals. PC-3 and 293T cells were obtained from the American Type Tissue Collection and maintained in DMEM supplemented with 10% fetal bovine serum and 1% antibiotics. Human umbilical vein endothelial cell (HUVEC) were kind gifts from Dr. Xingli Wang (Cardiothoracic Surgery Division of the Michael E. DeBakey Department of Surgery at Baylor College of Medicine Hospital) and cultured in ECGM medium supplemented with 20% fetal bovine serum, 1% BBE/heparin mixture, 1% antibiotics, and 0.5% fungizone (1). VEGF was from R&D Systems provided by Biological Resources Branch, National Cancer Institute-Frederick Cancer Research and Development Center. Matrigel was from BD Biosciences. Kp-10 peptide (purity >95%) was obtained from H.D. Biosciences or Genenmed Synthesis.

Fertilized chicken eggs were purchased from Shanghai Poultry Breeding Co. C57BL/6 mice were from the National Rodent Laboratory Animal Resources, Shanghai Branch in China. Severe combined immunodeficient (SCID/Ncr) mice were purchased from the National Cancer Institute.

Proliferation assay. Proliferation studies were carried out using the CellTiter96 AQueous One solution cell proliferation assay as described previously (1). Briefly, cells were plated at ~5,000 HUVECs or PC-3 cells per well in 96-well plate and allowed to adhere to the plate with different concentrations of Kp-10. The cells were incubated for 48 to 72 h and the AQueous One solution (Promega) was added to the samples and measured at 490 nm.

Migration and tube formation assays. Two types of cell migration assays were done using HUVECs. First, we examined cell migration in scratch assays as described previously (42). Cells cultured in 6-well culture dishes were scratched, washed with PBS, and cultured for 24 h. Cells migrated toward the wound regions were imaged and counted. Modified Boyden chamber migration assays were done as described previously (1). First, 4 × 10⁴ HUVECs were cultured onto gelatin-coated 8 µm pore size chambers (BD Bioscience), and the bottom well was filled with 10 ng/µL VEGF as the chemoattractant. Twenty-four hours after incubating chambers, cells were fixed with 4% formaldehyde and stained with H&E. Migrated cells were imaged and counted using Olympus IX70 inverted microscope (Olympus) connected to digital camera DMX1200. For tube formation assay, 4 × 10⁴ cells were cultured onto Matrigel-precoated 24-well culture dishes for 4 h at 37°C and determined as counting branching points and tubes. Each experiment was done four times and independently done in triplicate.

Chicken embryo CAM assay. According to previous method (43), embryonic eggs were incubated in 38.5°C to 39°C with the relative humidity at 65% to 70%. Five days later, a 1 to 2 cm² window was opened. The shell membrane was removed to expose the CAM. As the carrier, 6-mm-diameter Whatman filter disk, which absorbed Kp-10 peptide, was put on the CAM. Only PBS in the carrier was the control group. The window was sealed and eggs were incubated again. Four days later, the CAM was observed under stereomicroscope and the neovascularization was quantified. Assays for each test sample were carried out three times (n = 15-20).

Mouse corneal micropocket assays. The mouse corneal assay was done as described previously (44). Corneal micropockets were created with a modified von Graefe cataract knife in one eye of each 5- to 6-week-old C57BL/6 mice. Micropockets (0.25 ± 0.35 mm) of sucrose octasulfate-aluminum complex coated with hydron polyoxyethylenehydroxyethyl containing 160 ng VEGF165 or 1 µg Kp-10 plus 160 ng VEGF-165 were implanted into mouse corneal micropockets (n = 10-11). Seven days later, photos and data were obtained. The area of neovascular response, vessel length, and clock hours of new blood vessel were calculated according to the formula: area (mm²) = 0.2 × π × VL (mm) × CN (mm), where CN is the clock hours of neovascularization, 1 clock hour equals 30° of arc and VL is the maximal vessel length extending from the limbal vasculature toward the pellet.

Xenograft tumor growth assay and immunohistochemistry. PC-3 human prostate tumor cells (3 × 10⁶) were implanted s.c. on the back of 4-week-old male severe combined immunodeficient mice (n = 6). After 7 days, tumor volume reaching 50 mm³, Kp-10 (50 µg/50 µL/mouse) was injected intrathecally every day. Control groups were injected only with PBS. The growth of the tumor xenograft was evaluated in a pilot study by determining the tumor volume using digital caliper every 2 days. Tumor growth was measured as a following equation: length × width² / 2.25. Mice were continually observed until were killed. Tumors were removed and fixed and embedded with paraffin. Specific blood vessel staining was done on the 5 µm sections with CD31 antibody (1). Images were taken with Zeiss microscope. The number of blood vessels was counted.

Luciferase, electrophoretic mobility shift assays, chromatin immunoprecipitation assays, and reverse transcription-PCR. HUVEC was transfected with pVEGF165-luc, pVEGF165-luc, or pVEGF165-luc for 24 h and then subjected to the luciferase assay. For the luciferase assay, luciferase kit was used according to the manufacturer's protocol (Promega). Electrophoretic mobility shift assays were carried out as described previously (6). In brief, nuclear protein extracts (5 µg) were incubated with [γ-32P]ATP-radiolabeled double-stranded oligonucleotides. Chromatin immunoprecipitation assays were carried out as described previously (45) with 5 µg anti-Spi antibody (Santa Cruz Biotechnology) and rabbit preimmune serum. To detect VEGF mRNA expression, RNA was extracted from cells using Trizol (Invitrogen) and cdNA was amplified using reverse transcriptase. VEGF mRNA was amplified by general PCR. Primers are as follows: forward 5'-TGGCTTGTGCTCTACCTC-3' and reverse 5'-TCACCGCTCCTGTTGTAC-3'. Primers for glyceraldehyde-3-phosphate dehydrogenase used for loading control are as follows: forward 5'-GGATCGTGGAAGGACTCAT-3' and reverse 5'-ACACCTGTTGCTCACGTTA-3'.

Western blot and immunoprecipitation. Fifty micrograms of protein from cells lysed with radiomunoprecipitation assay buffer were loaded onto 8% to 12% SDS gel, transferred to polyvinylidene difluoride membrane, and incubated with the appropriate antibodies. Phosphorylated form of c-Src was detected using anti-c-Src pY416 antibody (Cell Signaling). To examine FAK phosphorylation, anti-FAK Y397 and anti-FAK Y576/577 (Cell Signaling) were used. Antibodies for Jun NH2-terminal kinase, phospho–Jun NH2-terminal kinase, phospho–extracellular signal-regulated kinase, extracellular signal-regulated kinase, phospho–p38, p38, VEGF, and actin were purchased from Santa Cruz Biotechnology. For the Rho GTPase activity, substrates such as GST-PK binding domain or GST-Rhotein binding domain induced in Escherichia coli were pulled down, incubated with GST-Sepharose beads, and then mixed with 500 µg whole protein. To detect active Rho GTapas, the appropriate antibody such as anti-RhoA, anti-Cdc42, or anti-Rac1 (all three from Santa Cruz Biotechnology) was used. Substrate-bound Rho GTapas present their active state.

Statistics. Statistical results in vitro and in vivo were calculated using the standard two-tailed Student’s t test on Microsoft Excel. Some statistical analyses of data were done using the two-way ANOVA followed by Bonferroni post hoc test using the GraphPad Quickcalc online Web site.

Results

Kp-10 inhibits HUVEC migration, invasion, and tube formation. To understand the function of Kp-10 in HUVECs, we examined the expression of GPR54, the endogenous receptor for Kp-10 in different cell lines. Our data indicate that GPR54 is expressed in HUVEC and N3 cells but not in Chinese hamster...
ovary, Cos7, NIH3T3, and HEK293T cells (Fig. 1A, left). As endothelial cell proliferation is important and necessary for angiogenesis, we investigated the inhibitory effect of Kp-10 on the growth of endothelial cells. Survival curves obtained with the MTS assay showed that high concentration of Kp-10 did not inhibit the proliferation of HUVECs (Fig. 1A, right), suggesting that Kp-10 has little effect on HUVEC proliferation at normal physiologic concentration. As cell migration and invasion are two key steps for endothelial cells to form new blood vessels during angiogenesis processes, we performed wound-healing assay and Boyden chamber migration and invasion assays to determine the effects of Kp-10 on HUVEC migration and invasion. Kp-10 inhibited the migration of HUVECs in a dose-dependent manner (Fig. 1B and C). In vitro, endothelial cells can spontaneously form a three-dimensional tubular capillary-like network on Matrigel culture. To examine the effect of Kp-10 on HUVEC tubule formation, we performed the tubule formation assays in the presence of different concentrations of Kp-10 peptide in Matrigel. As shown in Fig. 1D, Kp-10 dramatically inhibited HUVEC tube formation in a dose-dependent manner, suggesting that Kp-10 regulated angiogenesis in vitro.

The concentration of Kp-10 used in the angiogenesis study with HUVEC is much higher than that used in standard intracellular Ca\(^{2+}\) measurement. To make sure that Kp-10 used in our assay conditions are active, we performed intracellular Ca\(^{2+}\) measurement in both Chinese hamster ovary cells overexpressing GPR54 and HUVECs (endogenous GPR54). Our data indicate that Kp-10 activates intracellular Ca\(^{2+}\) in Chinese hamster ovary-GPR54 cells with an EC\(_{50}\) at 23 nmol/L, whereas the EC\(_{50}\) is \(\sim\) 225 nmol/L in HUVECs (Supplementary Fig. S1). These data indicate that Kp-10 is active and the effect of Kp-10 in HUVEC requires much higher concentration, which is similar to the effects we observed for Kp-10 in the inhibition of HUVEC angiogenic abilities.

Kp-10 suppresses angiogenesis in vivo. To determine the effects of Kp-10 on angiogenesis in vivo, we examined whether Kp-10 regulates angiogenesis using the CAM assays. As shown in Fig. 2A, Kp-10 inhibited chicken embryonic blood vessel formation at the concentration of 5 to 20 μg/disk. Within avascular areas
(circled area of 15 mm diameter with ~176 mm² around a filter paper disk), the number of newly formed blood vessels (black arrows) was significantly decreased in Kp-10–treated disk (Fig. 2A, left). The effect of Kp-10 is dose-dependent without any inflammation (Fig. 2A, right). These data suggest that Kp-10 suppresses angiogenesis in chicken embryonic blood vessel formation assays.

To confirm the antiangiogenic activity of Kp-10 in CAM assays, we further performed mouse corneal micropocket assay (Fig. 2B). Micropellets (pointed with arrows) coated with the slow-release polymer-hydron containing control buffer, VEGF, Kp-10, or VEGF and Kp-10 were implanted into the avascular corneas of C57BL/6 mice, respectively. VEGF strongly induce new blood vessel formation in mouse cornea (Fig. 2B, VEGF, left). Addition of 10 µg Kp-10 significantly inhibited VEGF-induced neovascularization in the cornea (Fig. 2B, VEGF + Kp-10, right). Quantitation of corneal neovascularization, presented as vessel length, clock hour (the proportion of the circumference vascularized when the eye is viewed as a clock), and vessel area showed that Kp-10 blocked VEGF-induced corneal neovascularization by 60% to 80% (Fig. 2C).

As for the controls, we have used PBS, a scrambled peptide, and the Kp-10 lacking the COOH-terminal amide in the experiments, the scrambled peptide has no effect on angiogenesis, whereas the Kp-10 without COOH-terminal amide has weak effect on angiogenesis at similar concentration in the assay (data not shown). In all treated mouse corneal experiments, no inflammation was observed and no weight loss or unusual behavior was detected, suggesting that Kp-10 has no or very little toxicity at the described experimental doses. These results suggest that Kp-10 significantly inhibits VEGF-induced angiogenesis in vivo.

**Kp-10 inhibits tumor angiogenesis and tumor growth.** To understand whether Kp-10 directly affects tumor cell growth, we performed PC-3 cancer cell proliferation assay. As shown in Fig. 3A, Kp-10 has little effect on tumor cell proliferation in vitro even at high concentration (100 µmol/L; P > 0.05), suggesting that Kp-10 had no direct effect on tumor cell proliferation. Given that tumor growth is angiogenesis-dependent and suppression of angiogenesis can inhibit tumor growth, we further examined the inhibitory function of tumor growth by Kp-10 using mouse xenograft model. As shown in Fig. 3B and C, after a 45-day treatment with Kp-10, the mean tumor volume of Kp-10–treated group were much less than that of PBS-treated control group (P < 0.01). In addition, there was little difference in body weight between control and Kp-10–treated mouse groups (Fig. 3C, right), suggesting little side effect of Kp-10.

![Figure 2](https://example.com/figure2.png)

**Figure 2.** Inhibition of angiogenesis by Kp-10 in vivo. A, Kp-10 inhibits angiogenesis in chicken embryo CAM assay. Representative pictures of PBS-control and Kp-10–treated CAM, showing inhibition of new blood vessel growth (black arrows) by the treatment of different concentrations of Kp-10 within a defined area surrounding the implanted disk. *, P < 0.05; **, P < 0.01. B, Kp-10 inhibits angiogenesis using mouse corneal micropocket assay. Micropellets (yellow arrows) containing 160 ng VEGF were implanted into corneal micropockets of C57BL/6 mice as described in Materials and Methods. The effects of Kp-10 on angiogenesis in vivo were examined using slow-release polymer containing four different treatment groups: saline alone, 1 µg Kp-10 alone, 160 ng VEGF alone, and 160 ng VEGF + 1 µg Kp-10. Saline alone and Kp-10 alone were used as controls in the experiments. Corneal neovascularization was measured and photographed with a stereomicroscope on day 7 after implantation. Arrows, positions of pellets. C, Kp-10 inhibited vessel length, clock hours of circumferential neovascularization, and area of neovascularization, respectively. The biomicroscopic assessment was conducted by two independent observers. Mean ± SE. **, P < 0.01.
in the mouse model. To verify the inhibitory effect of Kp-10 on tumor angiogenesis, we stained the 5 µm tumor sections with anti-CD31 antibody. The average vessel number in Kp-10–treated group was dramatically less than that in tumors of control group (Fig. 3D), indicating that Kp-10 significantly inhibits tumor angiogenesis and prevents tumor growth.

**Kp-10 decreases Sp1-dependent VEGF expression.** As the expression level of VEGF is important for tumor angiogenesis (3–5), we examined whether Kp-10 affects VEGF expression. As shown in Fig. 4A, Kp-10 decreased expression of both VEGF mRNA and protein in HUVEC. Thus, we further investigated whether Kp-10 regulated VEGF promoter activity in certain conditions such as hypoxia. As shown in Fig. 4B, Kp-10 not only suppressed the basal VEGF promoter activity but also decreased the activity of VEGF promoter (pVEGF2018-luc) in both hypoxia cells (CoCl2-treated cells) and hypoxia-inducible factor-1α–overexpressing cells, respectively (Fig. 4B, left). Because the VEGF promoter contains both hypoxia-inducible factor-1α–binding region and the GC-rich region or Sp1 binding, we next examined whether the regulation of VEGF promoter activity by Kp-10 is dependent on hypoxia stimulation. Using the two luciferase-reporting vectors containing the GC-rich regions where the hypoxia-inducible factor-1α–binding site is deleted (pVEGF133-luc or pVEGF85-luc), we show that Kp-10 significantly inhibited the activity of VEGF promoter (Fig. 4B, right), suggesting that Kp-10 regulated the expression of VEGF in a hypoxia-inducible factor-1α–independent manner.

As VEGF promoter contains high GC boxes to which Sp1 binds (8–11), we examined whether Kp-10 affected VEGF promoter binding of Sp1 using electrophoretic mobility shift assays and chromatin immunoprecipitation assays. As shown in Fig. 4C, Kp-10 at 1 µmol/L inhibited DNA binding of Sp1 to the VEGF promoter in electrophoretic mobility shift assay in both hypoxia and normal conditions (with or without the treatment of CoCl2). To further confirm the effect of Kp-10 on Sp1 binding of VEGF promoter, we perform chromatin immunoprecipitation assays using different
concentrations of Kp-10 in the treatment (Fig. 4D). Our data showed that Kp-1 inhibited the binding activity of Sp1 protein to VEGF promoter and suppressed Sp1-dependent VEGF expression in endothelial cells.

**Kp-10 down-regulates FAK- and Rac1/Cdc42-mediated migration signaling pathways in the endothelial cells.** We next analyzed Kp-10–inhibitory signaling pathways against VEGF-induced HUVEC migration during the processes of tumor angiogenesis. On VEGF stimulation, c-Src is phosphorylated and then interacts with FAK, which follows the activation of molecules downstream of c-Src/FAK complex. Thus, we examined whether Kp-10 affects VEGF-induced activation of c-Src/FAK complex. As shown in Fig. 5A, Kp-10 decreased VEGF-induced phosphorylation of c-Src at Y416 and inhibited c-Src–mediated phosphorylation of FAK at Y576/577, whereas Kp-10 has little effect on FAK autophosphorylation at Y397 (Fig. 5A).

To understand how Kp-10 regulates the downstream signaling molecules in HUVEC migration and invasion, we examine the activation of Rho GTPases, key molecular regulators in cell migration and invasion. As shown in Fig. 5B, Kp-10 inhibits the activation of Rac1 and Cdc42 by decreasing the amount of GTP-bound active forms of Rac1 and Cdc42 as measured in our pull-down assays. On the other hand, Kp-10 increased the activation of RhoA in a dose-dependent manner (Fig. 5B), suggesting that Kp-10 differentially regulated the activation of Rho family of GTPases. In addition, Kp-10 inhibited the phosphorylation of Jun NH2-terminal kinase but not extracellular signal-regulated kinase and p38 (Fig. 5C). Together, our data show that c-Src–mediated FAK phosphorylation and Rho GTPases are targets for Kp-10 inhibition of HUVEC migration and invasion.

**Discussion**

Previous reports showed that Kp-10 plays a role as vasoconstrictor, causing the narrowing of blood vessels. It is, however, unclear whether Kp-10 affects normal angiogenesis and tumor angiogenesis. In this study, we provide evidence that Kp-10 inhibits angiogenesis and tumor growth via blocking Sp1-dependent VEGF expression and suppressing VEGF-dependent FAK and Rac1/Cdc42 activation.

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**Figure 4.** Kp-10 decreases Sp1-dependent VEGF expression. A, Kp-10 inhibits VEGF expression in HUVECs. HUVECs were treated with different concentrations of Kp-10 for 24 h and then subjected to reverse transcription-PCR and Western blot analysis with anti-VEGF antibody. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and actin were used for PCR and Western blot loading controls, respectively. B, Kp-10 mediates the inhibitory effect through the GC-rich boxes in VEGF promoter regions. Left, HUVEC transfected with pVEGF2018-luc was treated with CoCl2 or hypoxia-inducible factor-1α in the presence or absence of 1 μmol/L Kp-10. At 24 h, luciferase assays were done. *, P < 0.05. Middle and right, HUVEC was transfected with pVEGF133-luc or pVEGF85-luc plasmid and treated with different concentrations of Kp-10 (10−2, 10−1, 1, and 10 μmol/L) for 24 h before luciferase activity was examined. *, P < 0.05; **, P < 0.01. C, Kp-10 inhibits the DNA-binding activity of Sp1 protein to VEGF promoter region in electrophoretic mobility shift assays. Kp-10 inhibits both hypoxia-independent and hypoxia-dependent Sp1 DNA-binding activity to VEGF promoter. When HUVEC was exposed to 1 μmol/L Kp-10 for 24 h, the binding of Sp1 to VEGF promoter region was diminished. The effects of Kp-10 on Sp1 DNA binding of VEGF promoter in hypoxic condition were measured with the addition of 100 nmol/L CoCl2. D, Kp-10 inhibits DNA-binding activity of Sp1 to VEGF promoter in vivo. Chromatin immunoprecipitation assays were done with different concentrations of Kp-10 after the cells were treated for 24 h.
Angiogenesis is a complex multistep process that involves cell proliferation, migration, and tube formation. It has been shown that suppression at any step of the processes in angiogenesis will inhibit the formation of new blood vessels (46). In the present article, we show that Kp-10 significantly inhibits HUVEC migration, invasion, and tube formation but not proliferation (Fig. 1). Furthermore, Kp-10 inhibited angiogenesis in vivo using CAM and mouse corneal micropocket assays (Fig. 2), suggesting that Kp-10 inhibited angiogenesis both in vitro and in vivo.

The Kp-10 concentration required to inhibit angiogenesis properties is much higher than that used in our intracellular Ca²⁺ signaling assays. Although we do not know the exact reasons for the much higher concentration of Kp-10 required for the inhibition of angiogenesis, the expression level of endogenous expressed GPR54 in HUVECs is much lower than that in Chinese hamster ovary cells overexpressing GPR54 in our Ca²⁺ measurement experiments and in the literature. In addition, HUVECs are large vessel endothelium, which makes them suboptimal for

**Figure 5.** Kp-10 inhibits the phosphorylation of c-Src/FAK and their downstream signaling proteins. A, Kp-10 decreased VEGF-activated phosphorylation of c-Src and FAK. Cells were treated with VEGF alone or VEGF + Kp-10 for 5 min. VEGF-dependent phosphorylation of c-Src and FAK was detected with anti-c-Src pY416, anti-FAK pY397, and anti-FAK pY576/577 antibody, respectively. B, Kp-10 down-regulates the activation of Rac1 and Cdc42 (GTP-binding) but activates RhoA in the GTPase pull-down assays. Cells were stimulated with the indicatives for 5 min. The activity assays of Rho GTPases were done using GST-PAK binding domain and GST-Rho kinase binding domain as described previously in our laboratory (34). C, Kp-10 decreased VEGF-induced phosphorylation of Jun NH₂-terminal kinase but has little effect on the phosphorylation of extracellular signal-regulated kinase 1/2 and p38 kinases. Phosphorylation of mitogen-activated protein kinases was examined using different antibodies after HUVECs were stimulated with VEGF alone or VEGF + Kp-10 for 15 min.

**Figure 6.** Diagram of Kp-10–mediated inhibition of tumor angiogenesis by suppressing Src/FAK and Rac1/Cdc42 signaling pathways and VEGF expression. Kp-10 activates GPR54 and Gαq signaling pathways and suppresses Sp1 binding of VEGF promoter and VEGF expression. At the same time, Kp-10–activated GPR54-G protein signaling axis regulates the phosphorylation and activation of Src and FAK as well as Rho GTPases (Rac1 and Cdc42), leading to the inhibition of cell migration and invasion. Down-regulation of VEGF expression and suppression of cell migration and invasion by addition of Kp-10 peptide lead to the inhibition of angiogenesis. Therefore, Kp-10 inhibits angiogenesis by suppressing Src-mediated FAK and Rac/Cdc42 signaling pathways and the expression of VEGF.
angiogenesis studies. Therefore, higher concentration of Kp-10 is required for inhibiting tumor angiogenesis, whereas lower concentration of Kp-10 can induce intracellular Ca²⁺ signaling pathway and has its normal physiologic functions in puberty.

Because tumor growth and metastasis depend on tumor angiogenesis, inhibition of tumor angiogenesis is a novel therapeutic modality toward controlling tumor metastasis (47). Kp-10 significantly inhibited the angiogenic tumor growth (Fig. 3B and C) but had little effect on tumor cell proliferation in vitro (Fig. 3A), suggesting that Kp-10 inhibits tumor growth not directly through inhibiting tumor cell proliferation per se but through inhibiting tumor angiogenesis. In addition, Kp-10–treated mice did not show any body weight loss compared with cisplatin-treated tumor-bearing mice, suggesting that Kp-10 has little toxicity compared with traditional antitumor drugs.

VEGF plays a key role in physiologic blood vessel formation and pathologic angiogenesis. In our studies, we show that Kp-10 inhibits Sp1-mediated VEGF expression in endothelial cells independently of the hypoxia condition (Fig. 4), suggesting that Kp-10 could block the initiation step of tumor angiogenesis by regulating the expression level of VEGF. Furthermore, Kp-10 inhibited endothelial cell migration and invasion via VEGF-mediated signaling, suggesting that Kp-10 targets multiple steps of tumor angiogenesis (Fig. 6).

In endothelial cells, VEGF activates c-Src and FAK and subsequently the formation of c-Src/FAK signaling complex. Our data showed that Kp-10 inhibited VEGF-induced phosphorylation of FAK (Fig. 5A). However, Kp-10 did not affect VEGF receptor phosphorylation (data not shown). Thus, Kp-10 via GPR54 appears to target VEGF receptor–induced activation of FAK in endothelial cells (Fig. 6). In addition, Kp-10 inhibits the activation of Rac1 and Cdc42 GTPases and Jun NH2-terminal kinase in the cells (Fig. 5B and C), indicating that Kp-10 inhibits key signaling molecules in cell migration and invasion (refs. 48–50; Fig. 6).

In conclusion, our data show that Kp-10 inhibits angiogenesis and suppresses tumor growth in vivo through inhibiting tumor angiogenesis by selectively blocking Sp1-dependent VEGF expression and by suppressing VEGF-mediated FAK and Rac1/Cdc42 activation in endothelial cells (Fig. 6). Our new finding of Kp-10 function in angiogenesis suggests a new role of kisspeptins as an antiangiogenesis agent.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References


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