Angioinhibitory Action of NK4 Involves Impaired Extracellular Assembly of Fibronectin Mediated by Perlecan-NK4 Association

Katsuya Sakai ‡, Takahiro Nakamura §, Kunio Matsumoto §, and Toshikazu Nakamura ‡ 1

From the ‡ Kringle Pharma Joint Research Division for Regenerative Drug Discovery, Center for Advanced Science and Innovation, Osaka University, 2-1 Yamadaoka, Suita, Osaka 565-0871, Japan; and the § Division of Tumor Dynamics and Regulation, Cancer Research Institute, Kanazawa University, 13-1 Takaramachi, Kanazawa 920-0934, Japan

Running title: Angioinhibitory mechanism of NK4
SUMMARY

NK4, a fragment of hepatocyte growth factor (HGF), exerts bifunctional action as a competitive antagonist against HGF and its receptor c-Met, and an angiogenesis inhibitor. Here we studied the anti-angiogenic mechanism of NK4. In cultured human endothelial cells, NK4 inhibited DNA synthesis induced not only by HGF but also by either basic fibroblast growth factor (bFGF) or vascular endothelial growth factor (VEGF). Even if c-Met expression was diminished by siRNA, NK4 inhibited bFGF-induced DNA synthesis, indicating that anti-angiogenic action of NK4 is c-Met-independent. Affinity purification with NK4-immobilized beads revealed that NK4 binds to perlecan. Consistent with this, NK4 colocalized with perlecan in endothelial cells. Perlecan is a multidomain heparan sulfate proteoglycan that interacts with basement membrane components such as fibronectin. NK4 inhibited extracellular assembly of fibronectin, by which fibronectin-dependent endothelial cell spreading was inhibited by NK4. Knockdown of perlecan expression by siRNA significantly abrogated the inhibitory effect of NK4 on fibronectin assembly and cell spreading. In NK4-treated endothelial cells, tyrosine-phosphorylation of focal-adhesion kinase and Rac activation were reduced, whereas overexpression of activated Rac recovered the DNA synthesis in NK4-treated endothelial cells. These results indicate that the association between NK4 and perlecan impairs fibronectin assembly, thereby inhibiting anchorage-dependent signaling. The identified mechanism for angiostatic action provides further proof of significance for NK4 in the treatment of cancer and potentially for vascular regulation as well.

INTRODUCTION

The manipulation of angiogenesis has potential therapeutic value for the treatment of a variety of diseases including cancer, arthritis, and cardiovascular disease (1, 2). In addition to endothelial cell migration and proliferation, angiogenesis is a process involving dynamic matrix transition (3). During angiogenesis, the vascular basement membrane undergoes proteolytic degradation and transit to the provisional matrix consisting of fibronectin, etc., followed by an intermediate and mature new vascular basement membrane. Growing evidence has shown that such an extracellular matrix (ECM) not only provides mechanical support to the cells, but also essentially regulates cell growth, migration, and survival. The fact that a number of endogenous inhibitors of angiogenesis have been identified from proteolytic fragments of ECM molecules also highlights the important regulatory roles of ECM in angiogenesis (3).

NK4 is a proteolytic fragment of hepatocyte growth factor, HGF (4), consisting of an N-terminal hairpin domain and four kringle domains of the α-chain of HGF (5). By competitively binding to HGF receptor c-Met, NK4 acts as an HGF-antagonist (5, 6). The NK4 fragment seems to be physiologically generated by mast cells and neutrophils peptidases during inflammation (7). Since HGF regulates malignant behavior in a variety of tumors by inducing invasive, angiogenic, and metastatic responses (8, 9), the blockade of HGF-c-Met signaling by NK4 is a strategy to inhibit tumor invasion and metastasis (6, 9-11). During investigation of a therapeutic approach with NK4 in experimental cancer models, we unexpectedly found that NK4 functions as an angiogenesis inhibitor (12). Based on the bifunctional characteristic as HGF-antagonist and angiogenesis inhibitor, NK4 suppressed malignant behavior of cancers, including invasion, metastasis and angiogenesis-dependent tumor growth (9-12).

The angiostatic activity of NK4 is probably independent of its original activity as an...
HUVECs were synchronized—Recombinant NK4 was prepared from TAKARA BIO. Human plasma fibronectin (Sigma) was biotinylated with NHS-sulfo-LC-LC-biotin (PIERCE) according to the manufacturer’s instruction. Anti-HGF antibody used for NK4 detection was prepared as described previously (13). Other antibodies were obtained as follows: Anti-Rb (G3-245), anti-Cyclin D1 (DCS-6), anti-Paxillin (349), and anti-5-Bromo-2′-deoxyuridine (BrdU) (B44), BD Biosciences; Anti-c-Met (C-12), anti-PAK (N-20), and anti-c-Myc (A-14), Anti-phosphotyrosine (PY99), Santa Cruz Biotechnology; Anti-β-Actin (AC-15) and anti-α-Tubulin (B-5-1-1), Sigma; Anti-phospho-ERK1/2 (E10), Cell Signaling; Anti-ERK 1/2, Upstate Biotechnology; Anti-phospho-FAK (pY397), Biosource; Anti-Fibronectin (3E3) and anti-Laminin γ1 (A5), Chemicon; Anti-Perlecan (7B5), Zymed Laboratories; Anti-Heparan sulfate (F58-10E4), Seikagaku; Anti-Syndecan-1, BioVision; and, Anti-Syndecan-2, R&D Systems.

**EXPERIMENTAL PROCEDURES**

Materials—Recombinant NK4 was prepared as described elsewhere (12). VEGF-A165 was obtained from R&D Systems. bFGF was a gift from Kaken Pharmaceutical. Fibronectin-related peptides (GRGDSP and GRGESP) were obtained from TAKARA BIO. Cycloheximide was obtained from Calbiochem. Human plasma fibronectin (Sigma) was biotinylated with NHS-sulfo-LC-LC-biotin (PIERCE) according to the manufacturer’s instruction. Anti-HGF antibody used for NK4 detection was prepared as described previously (13). Other antibodies were obtained as follows: Anti-Rb (G3-245), anti-Cyclin D1 (DCS-6), anti-Paxillin (349), and anti-5-Bromo-2′-deoxyuridine (BrdU) (B44), BD Biosciences; Anti-c-Met (C-12), anti-PAK (N-20), and anti-c-Myc (A-14), Anti-phosphotyrosine (PY99), Santa Cruz Biotechnology; Anti-β-Actin (AC-15) and anti-α-Tubulin (B-5-1-1), Sigma; Anti-phospho-ERK1/2 (E10), Cell Signaling; Anti-ERK 1/2, Upstate Biotechnology; Anti-phospho-FAK (pY397), Biosource; Anti-Fibronectin (3E3) and anti-Laminin γ1 (A5), Chemicon; Anti-Perlecan (7B5), Zymed Laboratories; Anti-Heparan sulfate (F58-10E4), Seikagaku; Anti-Syndecan-1, BioVision; and, Anti-Syndecan-2, R&D Systems.

**Cell Culture and Preparation of ECM**—Human umbilical vein endothelial cells (HUVECs) were purchased from Cambrex and grown in MCDB131 medium supplemented with 5% fetal bovine serum (FBS), 2 mM L-glutamine and 20 ng/ml bFGF. Culture plates for endothelial cells were coated with 0.1% gelatin (Sigma). For cell spreading assay, the cover glass was coated with 0.1% gelatin, 5 µg/ml fibronectin (Sigma), 5 µg/ml vitronectin (Sigma), 5 µg/ml collagen type I (BD Biosciences), 5 µg/ml laminin (Sigma), or 10 µg/ml collagen type IV (Sigma) at room temperature for 5 h. After washing with PBS twice, the cover glass was blocked with 3% BSA in PBS for 1 h. Human dermal fibroblasts were prepared as described elsewhere (12). ECM was prepared according to Hedman et al. (14). Conditioned medium was concentrated by centricon YM-100 (Millipore).

**BrdU Labeling**—HUVECs were synchronized in G0 by growth factor and serum deprivation for 24 h. G0-synchronized HUVECs were re-plated and cultured with MCDB131 medium supplemented with 5% FBS, 2 mM L-glutamine, 10 µM BrdU, ± 20 ng/ml of bFGF, VEGF, or HGF, and NK4 for 24 h. The cells were stained with anti-BrdU antibody and nuclei were stained with TO-PRO-3 (Invitrogen).

**Western Blot and Immunoprecipitation**—Cells were lysed on ice with 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 25 mM β-glycerophosphate, 50 mM NaF, 1 mM Na3VO4, 1% Triton X-100, 10% glycerol, and protease inhibitors (1 mM PMSF, 1 µg/ml of aprotinin, pepstatin A, and leupeptin). Protein concentration was determined by DC protein assay reagent (Bio-Rad).
Immunoprecipitation and western blotting was performed as described previously (12).

**Immunostaining**—For immunofluorescent staining, cells on cover glass were washed twice with PBS, fixed with 4% paraformaldehyde in PBS for 15 min, permealized with 0.2% Triton X-100 in PBS for 5 min, and blocked with 3% BSA in PBS for 1 h at room temperature. For BrdU staining, the cells were treated with 2 M HCl and neutralized with 0.1 M sodium tetraborate before blocking. The cells were incubated with primary antibodies, followed by secondary antibodies conjugated to Alexa Fluor-488, or -546 (Invitrogen). Biotinylated-fibronectin was stained with Alexa Fluor 488-conjugated streptavidin (Invitrogen). Actin was stained with phalloidin-rhodamin (Cytoskeleton). Nuclei were stained with TO-PRO-3. Cells were imaged at room temperature in Perma Fluor (Shandon Immunon) using a laser-scanning confocal microscope (LSM5 PASCAL; Carl Zeiss). Images were adjusted for brightness and contrast and cropped using Photoshop 6.0 (Adobe) software. The fluorescence intensity of the phospho-FAK (a), the area (b in pixel) in each focal adhesion, and the total number of focal adhesions per single cell (c) were determined by image analysis using adobe Photoshop 6.0. The fluorescence intensity of the phospho-FAK per cell was obtained by multiplying (a) x (b) x (c). Each value in the data represents the means ± SD calculated from more than twenty cells in random fields.

**Identification of Proteins Bound to NK4**—NK4 was biotinylated with NHS-sulfo-LC-LC-biotin. Plasma membrane from HUVECs was prepared by sucrose gradient centrifugation and was lysed with 20 mM Tris-HCl buffer (pH 7.5) containing 1% Triton X-100, 150 mM NaCl, 5 mM MgCl₂, 5 mM CaCl₂, and protease inhibitors as above. The lysate was incubated with 200 nM biotin-NK4 for 2 h at 4 °C and applied to streptavidin-agarose (Sigma). After washing with lysis buffer, proteins were eluted with 20 mM Tris-HCl (pH 7.5) containing 500 mM NaCl, concentrated by centrifcon YM-10 (Millipore), separated by SDS-PAGE, and visualized by silver staining. Gels were cut out, and proteins were reduced, S-carboxymethylated, and digested with trypsin. Peptides were analyzed by MALDI-TOF mass spectrometry (BRUKER DALTONICS).

**Binding Assay**—EIA/RIA 96-well plates (Coster, #9018) were coated with 0.2% gelatin in PBS overnight at 4 °C and blocked with 3% BSA in PBS at room temperature for 2 h. The wells were washed with PBS and incubated with indicated concentrations of biotin-fibronectin with or without 300 nM NK4 in PBS at room temperature for 2 h, washed with PBS, and incubated with 2 µg/ml Alexa Fluor 488-conjugated streptavidin in PBS at room temperature for 1 h. After washing with PBS, fluorescent intensity was measured with Multi-Detection Microplate Reader, POWERSCAN HT (Dainippon Pharmaceutical).

**Tubulogenesis in Collagen Gel**—HUVECs were suspended in 1 mg/ml type I collagen (BD Biosciences) at 1 x 10⁶ cells/ml. Cells were cultured in MCDB131 medium supplemented with 5% FBS, ± 20 ng/ml bFGF, ± 500 nM NK4 for 24 h. The cells were fixed with 4% paraformaldehyde in PBS and stained by 0.4% crystal violet. For detection of pericellular fibronectin, each 20 µL aliquot of cell suspension in collagen was dropped on cover glass. Cells were cultured for 24 h, fixed with 4% paraformaldehyde in PBS, and blocked with 3% BSA in PBS. The cells were incubated with primary antibody and subjected to DAB staining (Dako Cytomation). Between each step, samples were extensively washed 3 times with PBS for 1 h.
Small interference RNA (siRNA)—siRNA oligonucleotides were obtained from Nippon EGT. siRNA for c-Met (siMet#1: sense, 5'-CAUCAACAGUCCACUAGUUtt; anti-sense, 3'-ttGUAGUUGUACCGAGAUCAA-5'; siMet#2: sense, 5'-CUUCUUUGGUAGCAUACCtt; anti-sense, 3'-ttGAAGAAACAUCCGUUAUGG-5'). siRNA for perlecan (sense, 5'-GUUGGAGCAGCGGACAUAUtt; anti-sense, 3'-ttCAACCUCGUGCCGUUAUA-5'). Random siRNA was used as a control. HUVECs were transfected with 100 nM siRNA by lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions and recovered for 16 h in complete medium before each assay.

PAK Kinase Assay and Electroporation of Active Rho Family Proteins—PAK was immunoprecipitated and PAK activity was determined by measuring phosphorylation of myelin basic protein as described previously (15). cDNAs encoding Myc-tagged-Rho families (gifts from Dr. Y. Takai, Osaka Univ.) were cloned into pcDNA3.1(-)HisA. Cells (2 x 10⁶) resuspended in 400 µl Opti-MEM (Invitrogen) were transfected with 20 µg of each vector by electroporation at 240 V and 950 µF and recovered for 16 h in complete medium before each assay.

RESULTS

**c-Met-independent Inhibition of bFGF-induced DNA Synthesis by NK4**—We previously demonstrated that NK4 inhibited endothelial cell growth and migration induced not only by HGF, but also by either bFGF or VEGF (12). We first examined whether NK4 exhibits angioinhibitory action in other assays. In endothelial tube formation assay on matrigel, NK4 inhibited tube formation induced not only by HGF but also by bFGF (Supplementary Fig. S1). When we examined DNA synthesis by BrdU-incorporation, NK4 inhibited DNA synthesis induced not only by HGF, but also by either bFGF or VEGF (Fig. 1A). Thus, NK4 showed angioinhibitory action in different in vitro assays.

To address mechanism(s) by which NK4 inhibits angiogenesis, we evaluated intracellular signaling involved in DNA synthesis. NK4 dose-dependently inhibited the increase in cyclin D1 expression and Rb phosphorylation induced by bFGF in endothelial cells (Fig. 1B, left panels), suggesting that NK4 inhibits cell cycle progression at the G1 phase. Interestingly, NK4 did not inhibit bFGF-induced upregulation of cyclin D1 and Rb phosphorylation in fibroblasts (Fig. 1B, right panels). This was consistent with our previous finding that NK4 did not inhibit proliferation of fibroblasts enhanced by bFGF (12).

To clarify whether c-Met is required for the angioinhibitory action of NK4, we examined the effect of NK4 on DNA synthesis of endothelial cells in which c-Met expression was reduced by siRNA. Transfection of two different sequences of siRNA for c-Met remarkably inhibited c-Met expression in endothelial cells (Fig. 1C, right panel). In a comparable profile to that of control siRNA, bFGF-induced DNA synthesis was inhibited by NK4 in cells transfected with siRNA for c-Met (Fig. 1C, left graph). These results indicate that c-Met is not required for the inhibition of bFGF-induced DNA synthesis by NK4.

Identification of Perlecan as an NK4-binding Protein—To identify proteins mediating the angiostatic action of NK4, plasma membrane proteins prepared from endothelial cells were affinity-purified using beads immobilized with NK4. Proteins bound to the NK4-column were eluted, separated by SDS-PAGE, and subjected to protein staining. A mass spectrometry analysis of peptides revealed that these proteins were perlecan, laminin γ1 chain, and thrombospondin-1 (Fig. 2A,
upper panel). Western immunoblot of a purified fraction confirmed perlecan association (Fig. 2A, lower panel). We further examined whether NK4 is colocalized by immunostaining with perlecan on endothelial cells (Fig. 2B) and human fibroblasts (Supplementary Fig. S2A). NK4 significantly colocalized with perlecan both in endothelial cells and fibroblasts. However, the amount of NK4 colocalized with perlecan was much less due to very low expression of perlecan in fibroblasts (supplementary Fig. S2A and B). In contrast, significant colocalization of NK4 with thrombospondin-1 or laminin γ1 was not observed (unpublished data).

Inhibition of Fibronectin-dependent Endothelial Cell Spreading on Gelatin by NK4—Colocalization of NK4 with perlecan let us consider the effect of NK4 on the interaction between endothelial cells and ECM, because perlecan participates in the assembly of basement membrane components through its ability to bind ECM proteins including fibronectin (16-20). When endothelial cells were cultured on gelatin, NK4 induced morphological changes characterized by elongation and insufficient spreading (Fig. 3A). Because endothelial cell spreading on gelatin largely depended on the presence of serum, we speculated that the spreading on gelatin depends on the presence of either fibronectin or vitronectin in serum. To test this possibility, we examined the effect of GRGDSP peptides, blocking cell adhesion to fibronectin and vitronectin, on endothelial cell spreading on gelatin. GRGDSP peptides prevented endothelial cell spreading on gelatin, whereas control GRGESP peptide had no effect (Fig. 3B). Next, to deplete the fibronectin and vitronectin that was either in serum or produced by endothelial cells, endothelial cells were cultured with cycloheximide (CHX) under serum-free conditions. Under these conditions, endothelial cells did not spread on gelatin, whereas the addition of purified fibronectin restored the spreading (Fig. 3C). Importantly, NK4 inhibited this fibronectin-dependent endothelial cell spreading (Fig. 3C). We considered three possible mechanisms by which NK4 inhibited fibronectin-dependent endothelial cell spreading: 1) Inhibition of integrin-mediated endothelial cell adhesion on fibronectin; 2) Inhibition of interaction between gelatin and fibronectin; and, 3) Inhibition of extracellular assembly of fibronectin. Possibilities of 1) and 2) were unlikely, because i) endothelial cell adhesion on pre-coated fibronectin was not prevented by NK4 (Fig. 3D), and ii) the interaction between fibronectin and gelatin was not prevented by NK4 in the binding assay (Fig. 3E).

When we examined the effect of NK4 on endothelial cell spreading on other ECM substrates, NK4 did not inhibit endothelial cell spreading on either collagen IV, laminin, or vitronectin, while it moderately inhibited spreading on type I collagen (Supplementary Fig. S3). Thus, the inhibition of cell spreading by NK4 is most obvious on gelatin and moderately on type I collagen—both are major adhesive substances of fibronectin.

Inhibition of Extracellular Fibronectin Assembly—Fibronectin molecules are assembled by cells and exist as fibrillar extracellular matrix in tissues (21, 22). To address the possibility that NK4 may affect fibronectin assembly, we examined the extracellular fibronectin assembly by immunostaining (Fig. 4A). Endothelial cells were re-plated and cultured for 5 h in the presence or absence of NK4. Extracellular fibronectin assembly was observed in control cells, but it was mostly undetectable in the presence of NK4. Western immunoblot of fibronectin in deoxycholate-insoluble ECM or conditioned medium showed efficient deposition of fibronectin into ECM in the absence of NK4 (Fig. 4B). In the presence of NK4, fibronectin deposition in ECM
was remarkably reduced and unassembled fibronectin was detected in conditioned medium (Fig. 4B). Immunoblotting for laminin γ1 (LN) showed that the amount of LN in conditioned medium was not changed by NK4. These results indicate that NK4 inhibited fibronectin assembly into ECM without preventing the synthesis and secretion of fibronectin.

When endothelial cells were cultured in 3D collagen gel, bFGF induced tubular network formation over a period of 24 h, whereas this was significantly inhibited by NK4 (Fig 4C). Under these conditions, NK4 inhibited extracellular fibronectin assembly (Fig. 4D), which suggests that the impaired fibronectin assembly was associated with the inhibition of endothelial tube formation by NK4.

Perlecan Is Required for the Inhibition of Fibronectin Assembly and Cell Spreading by NK4—We speculated that the binding and colocalization of NK4 with perlecan might affect fibronectin assembly. Immunostaining of perlecan and fibronectin in endothelial cell culture showed colocalization of these molecules in extracellular assembly (Fig. 5A). Endothelial cells express other heparan sulfate proteoglycans (HSPGs), such as syndecans and glypicans (23), and syndecan-2 is involved in fibronectin assembly (21, 24). However, we did not observe colocalization of either syndecan-1, or -2 with fibronectin in endothelial cells (Supplementary Fig. S4). To test whether binding of NK4 to perlecan is required for its angiostatic action, we examined the effect of NK4 on endothelial cells in which perlecan expression was reduced by siRNA. Western immunoblot of cell lysates prepared after transfection of control-siRNA or perlecan-siRNA showed efficient knockdown of perlecan expression by perlecan-siRNA (Fig. 5B). NK4 significantly inhibited fibronectin assembly and cell spreading of endothelial cells transfected with control-siRNA (si-Control, Fig. 5C and D). In contrast, the inhibitory effect of NK4 on fibronectin assembly and cell spreading was significantly reduced in endothelial cells transfected with perlecan-siRNA (si-Perlecan, Fig. 5C and D). Taken together, these data suggest that perlecan-NK4 association is required for mediating the inhibitory effect of NK4 on fibronectin assembly and cell spreading.

Although we also examined whether the knockdown of perlecan can restore DNA synthesis and proliferation of endothelial cells treated with NK4, knockdown of perlecan resulted in defective DNA synthesis induced either by bFGF or by VEGF. This might be because of the essential role of perlecan as a co-receptor for heparin-binding growth factors (19, 20, 25).

Because NK4 has a heparin-binding feature (26), the heparan sulfate side chain of perlecan was a possible NK4 binding site. This was suggested because the addition of heparin almost completely prevented the binding of NK4 to the cells (Supplementary Fig. S5A), and this was associated with an abrogation of the inhibitory effects of NK4 on the fibronectin assembly (Supplementary Fig. S5A) and on endothelial cell spreading (Supplementary Fig. S5B).

Reduced Formation of Focal Adhesions by NK4 and Abrogation of the Inhibitory Effect of NK4 by Active Rac—Cooperation of signals from integrins and growth factor receptors, known as anchorage-dependency, is essential for proliferation of normal cells including endothelial cells (27). Inhibition of the extracellular fibronectin assembly and cell spreading strongly suggests that anchorage-dependent signal transduction was inhibited by NK4. Immunostaining of focal adhesions by paxillin and focal adhesion kinase (FAK) phosphorylated at Tyr 397 showed reduced numbers of focal adhesions with significantly
thinner and shorter morphology in NK4-treated cells (Fig. 6A). Reduced tyrosine phosphorylation of FAK and paxillin in NK4-treated cells were also detected by Western immunoblot (Fig. 6B).

Activation of Rac plays a critical role in integration of signaling pathways from growth factor receptors and integrin-mediated cell-ECM interaction in human endothelial cells (28). To analyze change in the activity of Rac, the activity of p21-activated kinase (PAK), a downstream target of Rac, was measured by in vitro kinase assay (Fig. 6C). Compared to cells kept in suspension, PAK activity increased in cells attached to a culture plate for 2 h, suggesting the activation of Rac by integrin-mediated anchoring of cells. However, NK4-treatment decreased PAK activity even when the cells were attached to a culture plate.

Next, we tested whether the expression of dominant-active Rac restores the endothelial cell DNA synthesis inhibited by NK4. For this purpose, activated forms of the Rho-family proteins Rac V12, Rho V14, and Cdc V12 were expressed in endothelial cells, and the cells were subjected to measurement of DNA synthesis (Fig. 6D and E). Similar expression levels of Myc-tagged Rac V12, Rho V14, and Cdc42 V12 in endothelial cells were confirmed by Western immunoblot (Fig. 6E, upper panel). In addition to nuclear staining of endogenous Myc seen in most cells, the cells expressing Myc-tagged Rac V12 were obvious from their cytoplasmic localization (Fig. 6D, arrows). Change in BrdU-incorporation was determined in cells expressing active Rho, active Cdc42, or active Rac under three conditions: without bFGF, with bFGF, and with bFGF+300 nM NK4 (Fig. 6E, lower graph). Expression of Rac V12 efficiently canceled the inhibitory effect of NK4 on bFGF-induced BrdU incorporation. In contrast, expression of activated forms of Rho and Cdc42 did not cancel the inhibitory effect of NK4. These results strongly suggest that the inhibitory effect of NK4 on anchorage-dependent Rac activity, which may be caused by impaired fibronectin assembly by NK4, plays a definitive role in the endothelial cell growth inhibition by NK4.

**DISCUSSION**

The HGF-c-Met pathway plays an important role in tumorigenesis, invasion-metastasis, and drug resistance (8, 9, 29-31). Likewise, anti-angiogenesis therapy has become standard for treatment of several types of advanced cancers. Therefore, the simultaneous inhibition of tumor angiogenesis and the HGF-c-Met pathway by NK4 seems to be a unique strategy for blocking the highly aggressive behavior of cancers. In experimental models, NK4 inhibited tumor growth, angiogenesis, and metastasis, and prolonged life span in several models (9-12). Together with a finding that NK4 seems to be generated in physiological or pathological conditions (7), understanding of the mechanism by which NK4 inhibits angiogenesis seems to provide not only a rationale for anti-cancer therapy by NK4 but also a possible physiological significance of NK4 in vascular regulation.

Both soluble growth factors and integrin-mediated adhesion to ECM are required for many cellular processes such as growth, survival and migration (27). In endothelial cells, the Rho-family GTPase Rac plays a critical role in the integration of signals from growth factor receptors and integrins required for cell cycle progression (28). We previously demonstrated that NK4 did not inhibit VEGF-induced activation of VEGF-receptor 2, and VEGF- and bFGF-induced activation of extracellular signal-related protein kinase (ERK) (12). Consistent with this, NK4 did not inhibit short-term and long-term activation of ERK 1/2 induced by bFGF or VEGF (Supplementary Fig. S6). These results suggest that NK4 inhibits growth
factor-induced cyclin D1 expression and Rb phosphorylation without preventing growth factor-induced receptor activation and ERK activation. Instead, NK4 reduced phosphorylation of FAK in focal adhesions and inhibited adhesion-dependent PAK kinase activity, and dominant expression of activated-Rac canceled inhibition of endothelial cell proliferation by NK4 (Fig. 6). Considering these results, we propose that NK4 inhibits anchorage-dependent signals leading to Rac activation that is required for endothelial cell proliferation.

Angiogenesis is a process involving dynamic ECM transition (3). During angiogenesis, the vascular basement membranes undergo proteolytic degradation and transit to the provisional matrix, followed by intermediate and mature new vascular BMs. Fibronectin is a major component of the provisional matrix and its expression is up-regulated on blood vessels during wound healing (32) and vascular sprouting (33). Furthermore, genetic or pharmacological inhibition of fibronectin-integrin α5β1 interaction disrupts angiogenesis in both developmental and pathological conditions (34-38), indicating an essential proangiogenic role of fibronectin and its main receptor integrin α5β1. Considering this essential proangiogenic role of fibronectin, we propose that the inhibition of extracellular fibronectin assembly in endothelial cells accounts for a mechanism by which NK4 inhibits angiogenesis.

Cells that have impaired proteoglycan synthesis exhibit a defective fibronectin matrix assembly (39). Among proteoglycans, the syndecans, transmembrane HSPGs that can bind to fibronectin, play a role in fibronectin assembly (40, 41). Syndecan-2, the major syndecan in fibroblasts, appears to have a regulatory effect on matrix assembly (24). However, significant colocalization of fibronectin with syndecan-1, -2 was not observed in HUVECs (Supplementary Fig. S4). Rather, colocalization of perlecan with fibronectin assembly was observed in HUVECs (Fig. 5A). Perlecan is expressed in basement membranes and is the major extracellular HSPG associated with blood vessels (19, 20). Perlecan provides binding sites for a number of ligands, such as basement membrane components including collagen type IV, nidogen, laminin, and fibronectin (16-20). The presence of perlecan in the basement membrane, and its ability to interact with other basement membrane components, suggests that it is involved in basement membrane assembly. Indeed, mice lacking perlecan showed reduced basement membrane integrity (42, 43). However, perlecan is not essential for fibronectin assembly in endothelial cells, because we observed that reduction of perlecan expression by siRNA did not prevent fibronectin assembly (Fig. 5C). We speculate that perlecan may provide a multidomain scaffold required for the proper organization of basement membrane components during remodeling from a fibronectin-rich provisional matrix to a mature basement membrane. The association of NK4 with perlecan may recruit NK4 to the site of cell-associated fibronectin assembly and may interfere with fibronectin assembly. The mechanism by which the specific interaction between NK4 and perlecan inhibits extracellular fibronectin assembly remains to be addressed.

NK4 inhibits proliferation of endothelial cells but neither fibroblasts nor canine renal epithelial cells (12). Here we show that inhibition of Rb phosphorylation and cyclin D1 expression by NK4 occurs in endothelial cells but not in fibroblasts (Fig. 1B). Because perlecan is a major component of the vascular basement membrane and is highly expressed in endothelial cells but not in fibroblasts (Supplementary Fig. S2), preferential expression of perlecan might be attributable to the cell
type-specific effect of NK4. Another possibility is the differences of ECMs that are required for cell proliferation in each cell type. For example, fibroblast proliferation was supported by integrin α1β1-mediated adhesion to collagen (44), while endothelial cell proliferation was supported by integrin α5β1 or αVβ3-mediated adhesion to either fibronectin or vitronectin (28).

NK4 consists of an N-terminal hairpin domain and four kringle domains of HGF. We have reported that only kringle domains of NK4 (K1-4) have anti-angiogenic effects, however, inhibition of endothelial cell growth by K1-4 was less potent than that by NK4 (45). N-terminal and 2nd-kringle domains are required for binding to heparin (26), and we show here that the anti-angiogenic effects of NK4 require binding to HSPGs. These results suggest the importance of the N-terminal domain, which might mediate the association of NK4 with HSPGs for the inhibitory effect of NK4 on endothelial cell proliferation. The recombinant N-terminal domain of HGF inhibited endothelial cell proliferation by preventing the binding of bFGF and VEGF to endothelial cells (46). This is likely due to the N-terminal domain simply occupying the glycosaminoglycan chain of HSPGs required for high affinity binding of these heparin-binding growth factors to growth factor receptors. However, this scenario is unlikely in the case of NK4, because NK4 inhibits endothelial proliferation, spreading, and fibronectin assembly without inhibiting growth factor signaling. Thus, although both the kringle and N-terminal domain of NK4 have independent angioinhibitory effects, both domains are required for the efficient inhibition of fibronectin assembly and proliferation by NK4.

In summary, we suggest that NK4 inhibits endothelial cell proliferation by interfering with fibronectin assembly and anchorage-dependent Rac activation required for cyclin D1 expression. Our proposal is that this process is dependent on the interaction of NK4 with HSPG, especially perlecan. HGF-c-Met and angiogenesis participate strongly in malignant progression of cancers. Thus, together with a newly identified mechanism for the angioinhibitory action of NK4, its bifunctionality (targeting both HGF-c-Met and angiogenesis) indicates a unique therapeutic value of NK4 in molecular-targeted therapy for cancer.
REFERENCES

FOOTNOTES

1 To whom correspondence should be addressed: Center for Advanced Science and Innovation, Osaka University, 2-1 Yamadaoka,Suita, Osaka 565-0871, Japan. Tel. and Fax: +81-6-6879-4130. E-mail: nakamura@casi.osaka-u.ac.jp

2 The abbreviations used are: HGF, hepatocyte growth factor; bFGF, basic fibroblast growth factor; VEGF, vascular endothelial growth factor; HSPG, heparan sulfate proteoglycan; siRNA, small interference RNA; FAK, focal adhesion kinase; ECM, extracellular matrix; BrdU, 5-bromo-2’-deoxyuridine; PAK, p21-activated kinase; ERK, extracellular signal-related protein kinase; HUVEC, human umbilical vein endothelial cell; FBS, fetal bovine serum; PMSF, phenylmethylsulfonyl fluoride; BSA, bovine serum albumin; CHX, cycloheximide; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; PBS, phosphate-buffered saline

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FIGURE LEGENDS

FIGURE 1. Inhibition of DNA synthesis by NK4 in a c-Met-independent manner. **A**, inhibition of DNA synthesis by NK4. G0-synchronized HUVECs were re-plated and cultured with indicated concentrations of NK4 and 20 ng/ml bFGF, VEGF, or HGF for 24 h. DNA synthesis was determined by BrdU-incorporation. Each value represents the means ± SD in a representative experiment from three independent experiments. **B**, changes in Cyclin D1 expression and Rb phosphorylation in HUVECs (left) and human dermal skin fibroblasts (right). G0-synchronized HUVECs or fibroblasts were re-plated and cultured with NK4, ± 20 ng/ml bFGF for 16 h. Total cell lysates (20 µg protein) were subjected to SDS-PAGE and subsequent immunoblotting with anti-Rb, anti-cyclin D1, or anti-actin antibody. pRb indicates phosphorylated Rb detected as upper band. **C**, inhibition of DNA synthesis by NK4 in endothelial cells in the absence or presence of c-Met expression. HUVECs transfected with indicated siRNA were synchronized in G0, re-plated and cultured with indicated concentrations of NK4, ± 20 ng/ml bFGF for 24 h. Right panel; Total cell lysates (400 µg protein) from HUVECs transfected with control siRNA (si-Cont) or siRNA for c-Met (si-Met#1, #2) were subjected to immunoprecipitation and immunoblotting using anti-c-Met antibody. Left graph; BrdU incorporation. Each value represents the means ± SD of three replicates in a representative experiment from two independent experiments.

FIGURE 2. Identification of perlecan as an NK4-interacting protein. **A**, identification of NK4-binding proteins by affinity purification and mass spectrometry analysis. Plasma membranes prepared from HUVECs were solubilized, incubated with or without biotinylated-NK4, and subjected to affinity purification using a streptavidin-agarose column. Bound proteins were subjected to SDS-PAGE and subsequent silver staining (upper panel). Each band was identified by trypsin digestion and MALDI-TOF MS analysis. Immunoblotting with anti-perlecan antibody confirmed perlecan in the NK4-bound fraction (lower panel). **B**, colocalization of NK4 with perlecan in endothelial cell culture. HUVECs were re-plated and cultured with 100 nM NK4 and 20 ng/ml bFGF for 2 h. Cells were fixed, permeabilized and stained for NK4 (red) and perlecan (green). Scale bar; 20 µm.

FIGURE 3. Inhibition of fibronectin-dependent endothelial cell spreading by NK4. **A**, effect of NK4 on endothelial cell spreading on gelatin. HUVECs were re-plated on gelatin-coated cover glass and cultured with medium containing 5% FBS, 20 ng/ml bFGF, ± 300 nM NK4 for 3 h. **B**, inhibition of endothelial cell spreading on gelatin by fibronectin related peptide. HUVECs were re-plated on gelatin-coated cover glass and cultured with medium containing 5% FBS, 20 ng/ml bFGF, ± 20 µM GRGDSP peptide or GRGESP peptide for 3 h. **C**, inhibition of fibronectin-dependent endothelial cell spreading by NK4. HUVECs were pre-treated with 100 µM cycloheximide (CHX) for 3 h, then re-plated on gelatin-coated cover glass and cultured with serum free-medium containing 100 µM CHX, 20 ng/ml bFGF, ± 10 µg/ml fibronectin (FN) or 300 nM NK4 for 3 h. **D**, effect of NK4 on endothelial cell spreading on fibronectin. HUVECs were re-plated on fibronectin-coated cover glass and cultured with medium containing 5% FBS, 20 ng/ml bFGF, ± 300 nM NK4 for 3 h. **A-D**, Cells were fixed, permeabilized and stained for paxillin (green) or actin (red). Scale bar; 50 µm. **E**, association between fibronectin and gelatin in the absence or presence of NK4. The binding of biotinylated-fibronectin to gelatin-coated wells was detected by Alexa Fluor 488-conjugated streptavidin and indicated as a relative fluorescent unit.
FIGURE 4. Inhibition of extracellular assembly of fibronectin by NK4. A, immunostaining of extracellular fibronectin assembly. HUVECs were re-plated and cultured in medium containing 5% FBS, 20 ng/ml bFGF, ± 300 nM NK4 for 5 h. Cells were fixed and extracellular fibronectin assembly was visualized by anti-fibronectin antibody before permealization. Scale bar; 20 µm. B, change in fibronectin accumulated in extracellular matrix (ECM) and conditioned medium (CM). ECM or CM were prepared from HUVECs cultured in medium containing 5% FBS, 20 ng/ml bFGF, ± 300 nM NK4 for 5 h (ECM) or 20 h (CM), subjected to SDS-PAGE, and immunoblotting for fibronectin (FN) or laminin γ1 (LN). C and D, Inhibition of endothelial cell tubulogenesis and extracellular fibronectin assembly in collagen gel by NK4. HUVECs were cultured in 1 mg/ml collagen gel in the presence of 20 ng/ml bFGF, ± 500 nM NK4 for 24 h. Cells were fixed and stained with crystal violet (C), or immunostained (D) with isotype control IgG or anti-fibronectin antibody followed by DAB staining (dark brown). Scale bar; 200 µm.

FIGURE 5. Requirement of perlecan for NK4-induced inhibition of extracellular fibronectin assembly and cell spreading. A, colocalization of perlecan with extracellular fibronectin assembly. HUVECs were re-plated and cultured in medium containing 5% FBS and 20 ng/ml bFGF for 2 h, then the medium was replaced with serum-free medium containing 20 ng/ml bFGF, 2 µg/ml biotin-fibronectin (biotin-FN) for 2 h. Cells were fixed and stained for biotin-FN (green) and perlecan (red) without permealization. B, inhibition of perlecan expression by siRNA. Total cell lysates (20 µg protein) from HUVECs transfected with control siRNA or siRNA for perlecan were subjected to SDS-PAGE and western immunoblot using anti-perlecan or anti-tubulin antibody. C, recovery of fibronectin assembly in the presence of NK4 by knockdown of perlecan expression. Forty-eight hours after siRNA-transfection, HUVECs were re-plated and cultured as described in (A). The cells were stained for biotin-FN and perlecan. D, requirement of perlecan for NK4-induced inhibition of cell spreading. Forty-eight hours after siRNA-transfection, HUVECs were re-plated and cultured in medium containing 5% FBS, 20 ng/ml bFGF, ± 300 nM NK4 for 5 h. Cells were fixed, permialized, and stained for paxillin (green) and actin (red). Scale bar; 50 µm.

FIGURE 6. Reduced anchorage-dependent signals by NK4, and abrogation of NK4-induced growth inhibition by dominant-active Rac. A, changes in distribution of focal adhesions and pFAK. HUVECs were re-plated and cultured in medium containing 5% FBS, 20 ng/ml bFGF, ± 300 nM NK4 for 3 h. Focal adhesions were stained for FAK phosphorylated at Y397 (pFAK, red) and paxillin (green). Scale bar; 50 µm. The right graph indicates change in fluorescence intensity of pFAK in focal adhesions per cell. The value was quantified as described in experimental procedures. The differences in values indicated by the double asterisk are statistically significant (**, P<0.01). B, immunoblotting of tyrosine phosphorylation in FAK and paxillin. Total Cell lysates (400 µg protein) were immunoprecipitated with anti-FAK antibody or anti-paxillin antibody. Immunoprecipitates were subjected to SDS-PAGE and immunoblotting with anti-phosphotyrosine antibody (pY). C, decrease in adhesion-induced PAK activity by NK4. Overnight serum-starved HUVECs were resuspended in serum-free medium containing 0.2% BSA and 20 ng/ml bFGF, and kept in suspension (Sus.) or attached (Att.) for 2 h in the presence or absence of NK4. Total cell lysates (300 µg protein) were immunoprecipitated with anti-PAK antibody in the presence or absence of PAK-epitope blocking peptides (BLK-pep) and subjected to kinase assay using myelin basic protein as a substrate (PAK activity) or immunoblotting using anti-PAK antibody (PAK protein). D, changes in DNA.
synthesis by expression of dominant-active Rac. HUVECs were transfected with Myc-tagged Rac V12 expression vector and subjected to measurement of bFGF-induced DNA synthesis by BrdU-incorporation in the presence or absence of 300 nM NK4. Cells were stained for Myc (red) and BrdU (green). Arrows indicate the cells expressing Rac V12 detected by cytoplasmic red staining. Nuclear red staining is due to endogenous c-Myc localization. Note that NK4-treatment reduced BrdU incorporation in cells lacking Rac V12, but not in cells expressing Rac V12. Scale bar; 20 µm. E, effect of Rac V12, Rho V14, or Cdc V12 expression on NK4-induced inhibition of DNA synthesis. Change in BrdU incorporation was determined in cytoplasmic Myc-positive cells under three conditions: the basal medium without bFGF (open bar), with bFGF (closed bar), with bFGF and 300 nM NK4 (hatched bar). Each value represents the means ± SD in a representative experiment from three independent experiments. Upper panel shows expression levels of Myc-tagged Rac V12, Rho V14, or Cdc42 V12 as determined by western immunoblot using anti-Myc antibody.

SUPPLEMENTARY DATA

SUPPLEMENTARY FIGURE LEGENDS

Supplementary FIGURE S1. Inhibition of endothelial cell tube formation on matrigel by NK4. A, appearance of tube formation. HUVECs were starved in 0.2% BSA/MCDB131 medium for 20 h. The cells were re-plated on 150 µl growth factor reduced matrigel (BD Biosciences) at 2.5 x10^4 cells/48-well plate. The cells were cultured in 400 µl of 0.2% BSA/MCDB131 medium, ± 10 ng/ml bFGF or HGF, ± NK4 for 20 h, and photographed. Scale bar; 200 µm. B, change in the number of areas closed by tubes. The number of areas closed by tubes was counted.

Supplementary FIGURE S2. Colocalization of NK4 with perlecan in human dermal fibroblast (A) and expression of perlecan mRNA (B). A, fibroblasts were re-plated and cultured with 100 nM NK4 for 2 h. Cells were fixed, permealized and stained for NK4 (red) and perlecan (green). Scale bar; 20 µm. B, total RNA was extracted by TRIZOL reagent (Invitrogen) from endothelial cells (HUVECs and HMVECs) and fibroblasts. First-strand cDNAs were synthesized by using SuperScript III Reverse Transcriptase (Invitrogen) with a random hexaprim. Perlecan mRNA was amplified by RT-PCR. Sequences for primers were as follows: Human perlecan (forward primer; 5’-ACAGTGCAACAAGTGCAAGG-3’, reverse primer; 5’-CTGAAGTGACCAGGCTCCTC-3’), Human GAPDH (forward primer; 5’-GAGTCAACCGGATTGTCGT-3’, reverse primer; 5’-GACAAGCTTCCCCGTTCTCAG-3’).

Supplementary FIGURE S3. Effect of NK4 on endothelial cell spreading on various ECMs. HUVECs were re-plated on ECM-coated cover glass and cultured with medium containing 5% FBS, 20 ng/ml bFGF, ± 300 nM NK4 for 3 h. Cells were fixed, permealized and stained for paxillin (green) or actin (red). Scale bar; 50 µm.

Supplementary FIGURE S4. Localization of extracellular fibronectin assembly, syndecan-1, and syndecan-2. HUVECs were re-plated and cultured in medium containing 5% FBS and 20 ng/ml bFGF for 2
h, then the medium was replaced with serum-free medium containing 20 ng/ml bFGF, 2 µg/ml biotin-fibronectin (biotin-FN) for 2 h. Cells were fixed and stained for biotin-FN (green) and syndecan-1 (S1, red) or syndecan-2 (S2, red) without permealization.

Supplementary FIGURE S5. Abrogation of inhibitory effect of NK4 on fibronectin assembly and cell spreading by heparin. A, inhibition of binding of NK4 to endothelial cells and recovery of fibronectin assembly by heparin. HUVECs were re-plated and cultured in medium containing 5% FBS and 20 ng/ml bFGF for 2 h, then the medium was replaced with serum-free medium containing 2 µg/ml biotin-fibronectin (biotin-FN), ± 300 nM NK4, ± 100 µg/ml heparin for 2 h. Cells were fixed, permealized and stained for biotin-FN and NK4. Arrows indicate extracellular fibronectin assembly. B, recovered spreading of HUVECs by heparin in the presence of NK4. HUVECs were re-plated and cultured in medium containing 5% FBS, 20 ng/ml bFGF, ± 300 nM NK4, ± 100 µg/ml heparin. Cells were stained for paxillin (green) and actin (red). Scale bar; 20 µm.

Supplementary FIGURE S6. Changes in MAPK activation induced by bFGF or VEGF. G0-synchronized HUVECs were re-plated and cultured with indicated concentrations of NK4, ± 20 ng/ml bFGF or VEGF, for 10 min (upper panels) or longer times (lower panels). Total cell lysates (20 µg protein) were subjected to SDS-PAGE and subsequent immunoblotting with anti-phospho-ERK1/2 or anti-ERK1/2 antibody. *; non-specific band.
Sakai et al. FIG. 1.

A

BrdU incorporation (%)

NK4 (nM) 0 0 100 300 1000 0 0 300 bFGF VEGF HGF

B

NK4 (nM) 0 0 100 300 1000 0 0 300 bFGF

pRb Rb
(105-115 kDa)

Cyclin D1
(35 kDa)

Actin
(42 kDa)

HUVECs Fibroblasts

C

bFGF-induced BrdU incorporation (%)

NK4 (nM) 0 0 500 1000

si-Cont si-Met#1 si-Met#2

IP; c-Met Blot; c-Met

(kDa) 175

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**Sakai et al. FIG. 2**

A

- Biotin-NK4
- Silver stain

**Blot:** Perlecan

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<th>(kDa)</th>
<th>175</th>
<th>125</th>
<th>100</th>
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**Perlecan**

Laminin γ1

Thrombospondin-1

B

- NK4
- Perlecan

**Merge**

Bar scale
A. Vehicle, NK4
B. Vehicle, RGDSP, RGEPR
C. - FBS, + CHX
   Vehicle, + FN, + FN, NK4
D. Vehicle, NK4
E. Bound biotin-FN (x1000 RU) vs. Biotin-FN (µg/mL)
   - None
   - NK4 (300 nM)
Sakai et al. FIG. 4

A  
Vehicle  
NK4  

B  
Blot:  
FN  
LN  
ECM  
CM  

C  
Vehicle  
bFGF  
bFGF+NK4  

D  
Vehicle  
NK4  

Control IgG  
Anti-fibronectin  

Vehicle  
NK4  

Control IgG  
Anti-fibronectin  

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Sakai et al. FIG. 5

**A**

- Biotin-FN
- Perlecan
- Merge

**B**

- Blot: Perlecan
- Blot: Tubulin
- (kDa)
- 175
- 47
- 32

**C**

- si-Control
  - biotin-FN
  - biotin-FN + NK4
- si-Perlecan
  - biotin-FN
  - biotin-FN + NK4

**D**

- si-Control
  - Vehicle
  - NK4
- si-Perlecan
  - Vehicle
  - NK4
**A**

![Image of cell cultures with BrdU incorporation](Vehicle, NK4)

**B**

- **Vehicle**
- **NK4**

<table>
<thead>
<tr>
<th>Relative fluorescence intensity of pFAK/cell</th>
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<tr>
<td><strong>Vehicle</strong></td>
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<td><strong>NK4</strong></td>
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**C**

<table>
<thead>
<tr>
<th></th>
<th>Sus.</th>
<th>Att.</th>
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<tr>
<td>BLK-pep</td>
<td>–</td>
<td>+</td>
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<tr>
<td>NK4 (nM)</td>
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<td>0</td>
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- **PAK activity**
- **PAK protein**
- **IgG**

**D**

- **Myc**
- **BrdU**
- **Merge**

<table>
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<tr>
<th></th>
<th>bFGF</th>
<th>bFGF + NK4</th>
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**E**

- **Mock**
- **Rac V12**
- **Rho V14**
- **Cdc V12**

<table>
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<tr>
<th>Blot: FAK (125 kDa)</th>
<th>Blot: Paxillin (88 kDa)</th>
</tr>
</thead>
</table>

- **IP: FAK**
- **IP: Paxillin**

**Graphs:**
- **BrdU incorporation (%)**
  - **Vehicle**
  - **bFGF**
  - **bFGF + NK4**

**Notes:**
- NK4 treatment affects BrdU incorporation and PAK activity.
- bFGF and NK4 together show increased BrdU incorporation.
Angioinhibitory action of NK4 involves impaired extracellular assembly of fibronectin mediated by perlecan-NK4 association
Katsuya Sakai, Takahiro Nakamura, Kunio Matsumoto and Toshikazu Nakamura

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