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HGF/NK4, a Four-Kringle Antagonist of Hepatocyte Growth Factor, Is an Angiogenesis Inhibitor that Suppresses Tumor Growth and Metastasis in Mice[1]

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ABSTRACT

We reported that NK4, composed of the N-terminal hairpin and subsequent four kringle domains of hepatocyte growth factor (HGF), acts as the competitive antagonist for HGF. We now provide the first evidence that NK4 inhibits tumor growth and metastasis as an angiogenesis inhibitor as well as an HGF antagonist. Administration of NK4 suppressed primary tumor growth and lung metastasis of Lewis lung carcinoma and Jyg-MC(A) mammary carcinoma s.c. implanted into mice, although neither HGF nor NK4 affected proliferation and survival of these tumor cells *in vitro*. NK4 treatment resulted in a remarkable decrease in microvessel density and an increase of apoptotic tumor cells in primary tumors, which suggests that the inhibition of primary tumor growth by NK4 may be achieved by suppression of tumor angiogenesis. *In vivo*, NK4 inhibited angiogenesis in chick chorioallantoic membranes and in rabbit corneal neovascularization induced by basic fibroblast growth factor (bFGF). *In vitro*, NK4 inhibited growth and migration of human microvascular endothelial cells induced by bFGF and vascular endothelial growth factor (VEGF) as well as by HGF. HGF and VEGF activated the Met/HGF receptor and the KDR/VEGF receptor, respectively, whereas NK4 inhibited HGF-induced Met tyrosine phosphorylation but not VEGF-induced KDR phosphorylation. NK4 inhibited HGF-induced ERK1/2 (p44/42 mitogen-activated protein kinase) activation, but allowed for bFGF- and VEGF-induced ERK1/2 activation. These results indicate that NK4 is an angiogenesis inhibitor as well as an HGF antagonist, and that the antiangiogenic action of NK4 is independent of its activity as HGF antagonist. The bifunctional properties of NK4 to act as an angiogenesis inhibitor and as an HGF antagonist raises the possibility that NK4 may prove therapeutic for cancer patients.

INTRODUCTION

HGF,[3] originally identified and cloned as a potent mitogen for hepatocytes (1, 2), plays both a biological and a physiological role in development and in tissue regeneration (3–5). In malignant tumors, HGF induces invasive, angiogenic, and metastatic responses through the c-Met/HGF receptor tyrosine kinase (6–10). In many carcinomas, HGF plays a role as a stroma-derived mediator in tumor-stromal interactions that confer invasion and metastatic potentials in cancer cells (10–12), whereas the generation of an autocrine HGF-Met loop is involved in the development of several types of tumors including sarcoma (13). Missense mutations in the *c-met* are causative genetic disorders in patients with sporadic and hereditary papillary renal carcinoma (14). These mutations resulted in the constitutive activation of Met and in the enhanced transformation of tumor cells (15), whereas HGF was also suggested to enhance the mutant Met-mediated transformation (16). Thus, blockade of HGF-Met signaling may be one strategy to inhibit tumor invasion and metastasis.

HGF is composed of the α chain, which contains a NH_2-terminal hairpin and four kringle domains and the catalytically inactive serine protease-like β-chain (2). Recently we prepared an antagonist for HGF by proteolytic digestion of HGF (17), and this HGF-antagonist, called HGF/NK4 (or NK4), is composed of the NH_2-terminal hairpin domain and subsequent four kringle domains of the α-subunit of HGF. NK4 binds to the c-Met/HGF receptor, but does not induce tyrosine phosphorylation of c-Met (17). NK4 competitively inhibits biological events driven by HGF-Met receptor coupling, including the invasion of distinct types of tumor cells (17, 18). On the other hand, NK1 and NK2, previously characterized HGF variants, have partial agonist and antagonist activity and elicit motility and the invasion of tumor cells and endothelial cells (19–22).

Angiogenesis, the formation of new blood vessels from preexisting blood vessels, is a critical process involved in embryonic development, tissue regeneration, and pathological conditions such as tumorigenesis and diabetic retinopathy (23, 24). Many investigators reported the essential role of angiogenesis during tumor progression (23, 25). Studies led to the thesis that angiogenesis is regulated by a balance between angiogenic and angioinhibitory factors (25, 26). In the activated endothelium, angiogenic growth factors predominate, whereas vascular quiescence is achieved by the dominance of angioinhibitory factors including angiostatin, endostatin, thrombospondin, platelet factor IV, the NH_2-terminal fragment of prolactin, etc. (25). Physiological and pathophysiological roles of angioinhibitory factors, as well as mechanisms by which these polypeptides inhibit angiogenesis, are largely unknown; however, several angiogenesis inhibitors have been shown to inhibit tumor growth and metastasis (27–30), and there are angiogenesis inhibitors under clinical trials for cancer treatment (29–32).

We now have evidence that NK4 is an angiogenesis inhibitor. NK4 not only antagonizes HGF-induced angiogenesis but also abrogates the angiogenesis induced by other angiogenic inducers. The antiangiogenic activity of NK4 is likely to be exhibited through a mechanism distinct from its initially characterized potential to act as an HGF-antagonist: the blockade of HGF-c-Met coupling. We report here that NK4 suppresses tumor angiogenesis, growth, and metastasis in mice.

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[3] The abbreviations used are: HGF, hepatocyte growth factor; EBM-2, endothelial basal medium; FBS, fetal bovine serum; MDCK, Madin-Darby canine kidney; CAM, chick chorioallantoic membrane; bFGF, basic fibroblast growth factor; VEGF, vascular endothelial growth factor; KDR, human Flk-1; MAPK, mitogen-activated protein kinase; LLC, Lewis lung carcinoma; Jyg-MC, Jyg-MC(A) murine mammary carcinoma; PCNA, proliferating cell nuclear antigen; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; HMVEC, human dermal microvascular endothelial cell.

MATERIALS AND METHODS

Materials. Human recombinant HGF was purified from the conditioned medium of Chinese hamster ovary cells transfected with human HGF cDNA (2, 33). Polyclonal anti-human HGF antibody (1 μg/ml) completely neutralized the biological activities of 1 ng/ml HGF (17, 18). Human recombinant bFGF and VEGF165 were obtained from R & D Systems (Minneapolis, MN). Human NK4 was prepared as described elsewhere (17). The level of endotoxin in the purified NK4 was determined to be 0.43–1.02 ng/mg using a *Limulus* amebocyte lysate kit from BioWhittaker. Accordingly, the level of endotoxin in endothelial growth media containing 1000 nM NK4 (the maximum dose in our system) was <0.067 ng/ml. This amount of endotoxin alone had no cytotoxic effects on endothelial cells [not shown and as described (34)].

Cell Culture. Human adult dermal microvascular endothelial cells and human lung-derived microvascular endothelial cells were purchased from Clonetics (San Diego, CA) and grown in EBM-2 supplemented with 5% FBS and endothelial cell growth supplements (Clonetics). Human pulmonary artery endothelial cells, purchased from KURABO (Osaka, Japan), were cultured in endothelial growth medium (EGM; KURABO). Rat coronary endothelial cells were isolated as described (35). Culture plates for endothelial cells were coated with 0.1% gelatin (Difco, Detroit, MI). Human dermal fibroblasts initially proliferated outward from dermal tissue obtained during plastic surgery. These cells were used within passage number 4–8. MDCK epithelial cells were a generous gift from Dr. R. Montesano (University of Geneva, Geneva, Switzerland). NIH3T3 fibroblasts, LLC cells, and Jyg-MC cells were obtained from RIKEN Cell Bank (Tsukuba, Japan). Rat coronary endothelial cells, human fibroblasts, MDCK, and NIH3T3 cells were cultured in DMEM supplemented with streptomycin, penicillin, and 10% FBS.

Cell Proliferation, Migration, and Invasion Assay. Endothelial cells were plated at 5×10^3 cells/cm^2 onto gelatinized 24-well tissue culture plates and cultured for 24 h. The medium was replaced with 0.5 ml of EBM-2 containing 5% FBS, and cells were cultured in the absence or presence of NK4, 10 ng/ml HGF, 3 ng/ml bFGF, 10 ng/ml VEGF, or their combinations. After 72 h, cells were dispersed by trypsin and counted by Coulter counter. To measure the proliferation of nonendothelial cells, human dermal fibroblasts, LLC cells, and Jyg-MC cells were plated at 5×10^3 cells/cm^2, whereas NIH3T3 and MDCK cells were plated at 2.5×10^3 cells/cm^2, on 24-well plates and cultured for 24 h. The medium was replaced with DMEM supplemented with 5% FBS and 10 ng/ml bFGF, test samples were added, and the cells were then cultured for 72 h.

Migration of endothelial cells was evaluated using a modified Boyden chamber assay, as described (12, 36). The cells were serum-starved in EBM-2 medium for 12 h and plated at 12×10^4 cells/cm^2 onto the polycarbonate filter with 5-μm pores (Costar, Cambridge, MA) coated with 13.4 μg/ml fibronectin (Orgagnon Teknika Corp., West Chester, PA). Test samples were added to the medium in the outer cup, and the cells were cultured for 5 h. The number of the cells which migrated to the undersurface of the filter was quantified by counting cells in five randomly selected microscopic fields (×200) in each well. *In-vitro* invasion of carcinoma cells was measured using a Matrigel invasion chamber (Becton Dickinson, Bedford, MA), as described (12).

Immunoprecipitation and Western Blot. Tyrosine phosphorylation of c-Met or KDR was analyzed as described elsewhere (18). Briefly, human adult dermal microvascular endothelial cells were grown on 100-mm plates and serum-starved overnight before treatment for 10 min with various concentrations of NK4 with or without bFGF, VEGF, or HGF (10 ng/ml each). Cell lysates were prepared, and equivalent amounts of protein were incubated overnight with a monoclonal antibody against phosphotyrosine (PY99; Santa Cruz Biotechnology, Santa Cruz, CA) and then incubated for 2 h with protein G-Sepharose. The immunoprecipitates were separated by 6% SDS-PAGE and electroblotted onto polyvinylidene difluoride membranes, and the proteins were probed with anti-c-Met antibodies (C-12; Santa Cruz Biotechnology) or anti-KDR/Flk-1 antibodies (C-1158; Santa Cruz Biotechnology). Immunoreactive bands were visualized by the enhanced chemiluminescence system (Amersham). For detection of phosphorylated ERK1/2 (p44/42 MAPK), total cell lysates were separated by 12% SDS-PAGE and transferred to polyvinylidene difluoride membranes. Blots were developed with phosho-specific antibodies against ERK1/2 (New England Biolabs, Beverly, MA). After being "stripped," the membranes were reprobed with antibody against ERK1/2 (Santa Cruz Biotechnology) as a loading control.

Tumor Study in Mice. Male nude mice 6- to 8-weeks-old (BALB/c nu/nu; Japan SLC, Inc., Hamamatsu, Japan) were s.c. implanted with 1×10^6 LLC cells or 5×10^6 Jyg-MC cells. After 4 days, an osmotic pump (Alzet 2002; Alza Corp., Palo Alto, CA) containing NK4 or BSA or saline alone was surgically implanted near the tumor, and NK4 or BSA solution or saline alone was continuously infused for 10 or 14 days into the s.c. region near the tumor mass. The size of tumors in all groups was measured using a dial caliper, and the volume of tumors was determined using the formula width^2 × length × 0.52. To analyze external lung metastases, mice were infused with NK4 solution or saline alone for 14 days, as described above, then the mice were killed on day 28 after tumor implantation.

Tumor tissues were fixed in Carnoy's fixative for 4 h or overnight in 70% ethanol at 4°C and embedded in paraffin according to standard histological procedures. For blood vessel staining, tissue sections fixed in Carnoy's fixative were pretreated with 5 μg/ml Proteinase K at 37°C for 15 min and incubated with antibody against von Willebrand factor (Dako, Glostrup, Denmark). The sections were sequentially incubated with biotin-labeled secondary antibodies and avidin-biotin peroxidase complex, as described (37). The number of blood vessels was counted under a light microscope at ×200 magnification using at least 20 randomly selected fields per section. For detection of proliferating cells or apoptotic cells, tissue sections fixed in 70% ethanol were analyzed as described elsewhere (18). Statistical analyses were performed with unpaired Student's t test (two-tailed). $P < 0.05$ was considered to be statistically significant.

***In Vivo* Angiogenesis Assay.** Antiangiogenic activity of NK4 on CAM was assayed, as described (29, 38). Briefly, fertilized white Leghorn chicken eggs were incubated at 37°C for 5 days, and a methylcellulose disk containing test material was placed within a sterilized silicon ring on CAM (38). The eggs were incubated at 37°C for 2 days. The white fat emulsion (Intralipos; WelFide Co., Osaka, Japan) was injected into the chorioallantois, and the vascular networks in the CAMs were blindly scored by two independent investigators for the presence or absence of an avascular zone (>3 mm in diameter) surrounding the implant. Statistical analyses were performed with the Fisher's exact probability test. $P < 0.05$ were considered to be statistically significant.

The antiangiogenic activity of NK4 was also assayed in the rabbit cornea, as described (39–41). Slow-releasing pellets were prepared by incorporating HGF, bFGF and/or NK4 into a 20-μl casting solution of an ethylene-vinyl acetate copolymer (EV40) in 5% methylene chloride. A microsurgical pocket (2 × 4 mm) was produced in the lower half of each eye of male albino Japanese rabbits such that a peripheral pocket ended at 2 mm from the limbus. A single pellet was deposited in the bottom of the pocket. Eyes were examined in a blind manner by slit-lamp microscopy every other day. An angiogenic response was scored positive when blood vessels from the limbal plexus occurred and capillaries progressed to reach the implanted pellet within 6–8 days, as described (40). Statistical analysis was performed with the χ^2 test. $P < 0.05$ was considered to be statistically significant.

Data Analysis. Statistical analyses were performed with unpaired Student's t test (two-tailed) unless mentioned otherwise in the text. Differences were considered to be statistically significant at $P < 0.05$.

RESULTS

Suppression of Tumor Growth, Angiogenesis, and Metastasis by NK4. We demonstrated previously that NK4 inhibits HGF-mediated growth and invasion of human carcinoma cells (18). In the present study, we attempted to elucidate the antagonistic effect of NK4 on metastasis in murine tumor models. For this purpose, we selected two metastatic murine tumors, LLC and Jyg-MC mammary carcinoma, because these cell lines express the c-Met/HGF receptor (not shown). *In vitro*, HGF, bFGF, and NK4 had no effect on proliferation and survival of these tumor cells (Fig. 1*A*, and not shown). On the other hand, both HGF and bFGF stimulated invasion of tumor cells, whereas NK4 specifically antagonized the invasion of these tumor cells induced by HGF but not by bFGF (Fig. 1*B*). When LLC and Jyg-MC cells are inoculated s.c. into athymic mice, both tumors formed nodules (20–40 mm^3 in volume) 4 days after implantation,

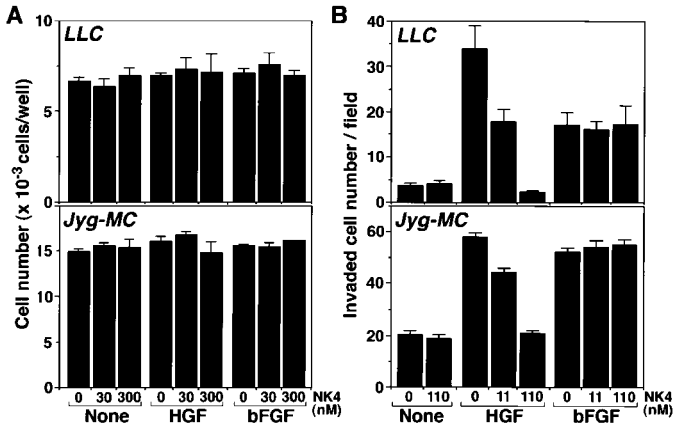

Fig. 1. Effects of NK4 on proliferation and invasion of LLC cells and Jyg-MC cells *in vitro*. *A*, effect of HGF, bFGF, NK4, and their combinations on proliferation of tumor cells. *B*, inhibitory effect of NK4 on invasion of tumor cells through Matrigel in the absence or presence of 10 ng/ml (110 pM) HGF or 10 ng/ml (550 pM) bFGF. Each value represents the mean ± SE.

and metastatic nodules in lung surface become visible 3–4 weeks after implantation.

To test the effect of NK4 on lung metastasis, NK4 or vehicle (saline alone or with BSA as a protein control) was infused continuously for 2 weeks, using an osmotic minipump, from the 4th day after tumor implantation. The mice were autopsied and the number of metastases in the lung surface was counted on the 28th day after the implantation. Despite the failure of NK4 to suppress tumor cell growth *in vitro*, continuous infusion of NK4 dose-dependently inhibited the growth of LLC primary tumors. The volume of NK4-treated tumors was inhibited to 62.5% at 10 μg/day ($P < 0.05$) and 30.1% at 25 μg/day ($P < 0.001$) on the 28th day as compared with findings in control tumors ($n = 4$/group; Fig. 2*A*). Lung metastasis was strongly suppressed by NK4-treatment in a dose-dependent manner; the number of lung metastases in control mice (12.9 ± 1.35) decreased to 54.8% ($P < 0.05$) and 9.46% ($P < 0.0001$) in NK4-treated mice at 10 μg/day and 25 μg/day, respectively (Fig. 2*B*). Similarly, NK4 (25 μg/day) also suppressed primary tumor growth and lung metastasis of Jyg-MC to 53.6% ($P < 0.05$) and 35.3% ($P < 0.05$), respectively ($n = 4$/group; Fig. 2). Infusion of BSA in saline at 25 μg/day for 2 weeks did not significantly change the tumor volume and number of lung metastases of both tumors (not shown). Although NK4 inhibited tumor metastasis, the result raised the question as to how NK4 suppresses growth of these primary tumors, inasmuch as HGF and NK4 have no direct effect on the proliferation of tumor cells.

To address this question, LLC primary tumors were excised on the 14th day after implantation (10 days after NK4 or saline administration), and the proliferation index of tumor cells *in situ* was quantified by immunohistochemistry of PCNA. Although the volume of primary tumors in NK4-treated mice (NK4 reached 10.4 ± 1.41 ng/ml in the blood in NK4-treated mice) decreased to 43% of the control ($n = 6$; $P < 0.01$), the proliferation index was not significantly changed by NK4-treatment; this was consistent with *in vitro* results (Fig. 3). Thus, we speculated that NK4 suppresses tumor growth by inhibiting the host compartment, such as angiogenesis, rather than the tumor-cell compartment. To examine this possibility, we measured the microvessel density in tumor tissues by von Willebrand factor immunostaining. The number of von Willebrand factor-positive vessels in NK4-treated tumors decreased to 55.8% of that of control tumors ($P < 0.001$), and some tumor vessels in NK4-treated mice were disrupted and had a short diameter (Fig. 3). Furthermore, TUNEL assay showed that NK4-treatment led to a 2.25-fold increase in the number of apoptotic cells ($P < 0.001$; Fig. 3). These findings are consistent with previous studies which showed that an angiogenesis inhibitor suppresses tumor growth by increasing apoptosis of tumor cells (37, 42). Thus NK4 seems to suppress primary tumor growth mainly through inhibition of tumor angiogenesis.

Inhibition of Endothelial Cell Growth and Migration by NK4. We next investigated the effect of NK4 on proliferation of human dermal microvascular endothelial cells. During a 3-day culture, HGF stimulated cell growth to a 2-fold-higher level over controls (without growth factor), and NK4 dose-dependently inhibited endothelial cell proliferation. Such inhibitory effects of NK4 were likely attributable to HGF-antagonist activity (43). However, NK4 also significantly abrogated the stimulatory effects of both bFGF and VEGF on endothelial cell proliferation, and the increase in cell number was blocked by 1000 nM NK4. Likewise, in the absence of growth factors, the number of cells increased to a 1.5-fold-higher level, presumably by

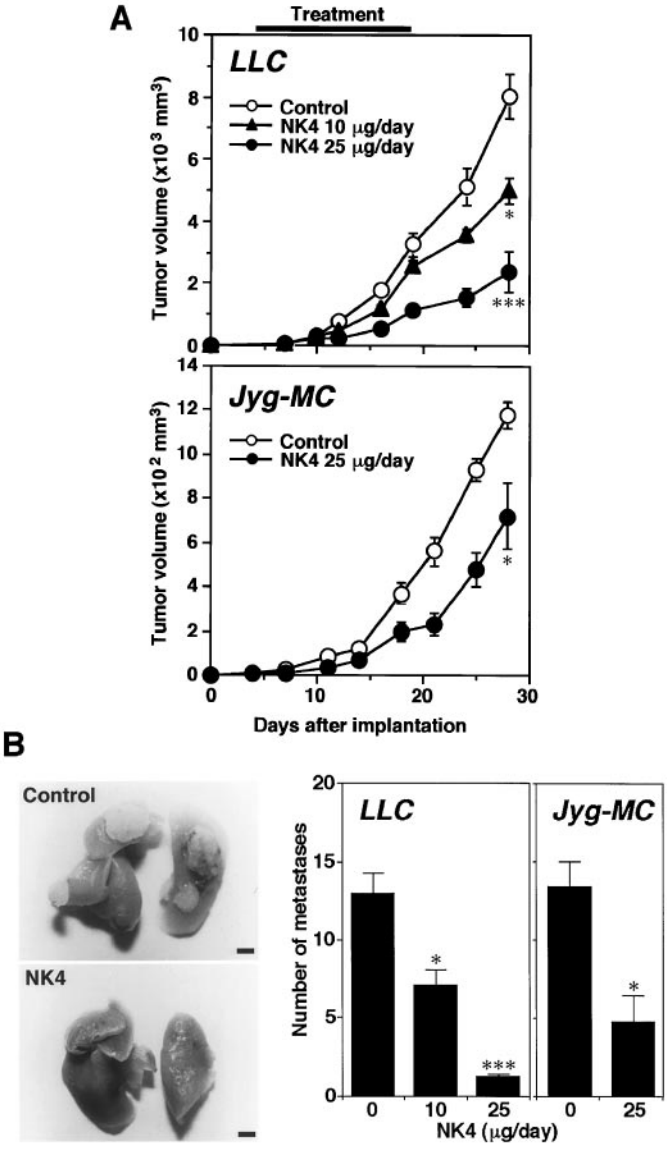

Fig. 2. Suppression of primary tumor growth and lung metastasis of LLC and Jyg-MC by NK4. *A*, inhibition of primary tumor growth by NK4. *B*, suppression of lung metastasis by NK4. Photographs show lungs of LLC-bearing mice. *Bars*, 2 mm. LLC cells or Jyg-MC cells were s.c. implanted in mice, and NK4 solution or saline was infused continuously for 14 days from the 4th day after tumor implantation ($n = 4$/group). At 28 days after tumor implantation, mice were autopsied and the number of metastases in the lung was measured. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Each value represents the mean ± SE.

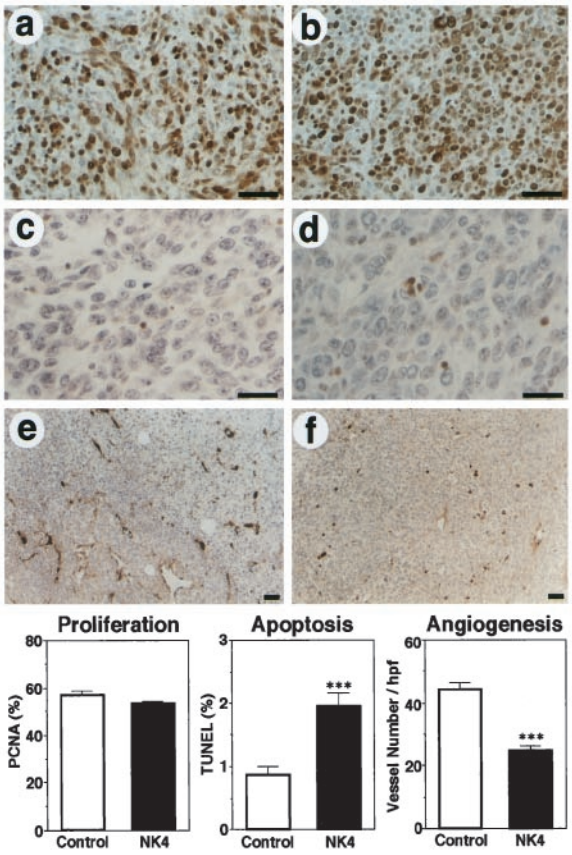

Fig. 3. Inhibitory effect of NK4 on tumor angiogenesis of LLC in mice. *Photographs* show a typical immunohistochemical appearance in tumor tissues from control saline-infused mice (*a*, *c*, and *e*) and NK4-infused mice (*b*, *d*, and *f*). Proliferation, apoptosis, and angiogenesis were detected using anti-PCNA antibody (*a* and *b*), a modified TUNEL method (*c* and *d*), and anti-von Willebrand factor antibody (*e* and *f*), respectively. *Bars*, 50 μm. *Graphs* show change in PCNA-positive cells, TUNEL-positive cells, and blood vessel number in the tumor tissue. Four days after the implantation of LLC cells, NK4 (25 μg/day) or saline alone was continuously injected into the s.c. region near the tumor mass using an osmotic pump. Ten days after NK4 treatment, tumors were resected and examined histologically (n = 6/group). Data represent the mean ± SE; ***, $P < 0.001$.

5% FBS; however, the basal increase in cell number was inhibited by 1000 nM NK4. Heat-treated NK4 (100°C, 10 min) lost inhibitory activity in regard to endothelial growth.

To determine whether NK4 has similar inhibitory effects on other endothelial and nonendothelial cells, we examined the effects of NK4 on the proliferation of several types of cells (Fig. 4*B*). Three distinct types of endothelial cells (human lung-derived microvascular endothelial cells, human pulmonary artery endothelial cells, and rat coronary endothelial cells) were cultured in the presence of bFGF with or without NK4. Dose-dependent growth inhibition by NK4 was seen in these three types of endothelial cells, whereas effectiveness differed somewhat for each cell type; the most potent inhibitory effect was seen with human microvascular endothelial cells. A similar inhibitory effect of NK4 was seen when these endothelial cells were cultured in the presence of VEGF (not shown). On the other hand, the proliferation of nonendothelial cells (human dermal fibroblasts, NIH3T3 mouse fibroblasts, MDCK canine renal epithelial cells), which were stimulated by bFGF, was not suppressed by NK4 at all (Fig. 4*B*). Likewise, NK4 had no inhibitory effect on proliferation of these nonendothelial cells stimulated by 10% FBS (not shown).

Because migration of endothelial cells, as well as cell proliferation, is an essential component responsible for angiogenesis, we next examined the effects of NK4 on the cell migration of human dermal microvascular endothelial cells (Fig. 4*C*). Endothelial cells were seeded on a filter membrane coated with fibronectin, and the number of cells that migrated through the membrane was measured. Migration of endothelial cells was stimulated by bFGF, VEGF, and HGF, whereas migration of the endothelial cells driven by these growth factors was strongly inhibited by NK4, mostly to the basal level at 300 nM NK4. Taken together, NK4 has an inhibitory effect on endothelial cell growth and migration stimulated by bFGF and VEGF as well as by HGF, and the antiproliferative activity is likely to be specific to endothelial cells.

Effects of Neutralizing HGF-antibody on Endothelial Growth and Migration. Although NK4 inhibited endothelial growth and migration stimulated by bFGF and VEGF, its antagonizing activity for HGF might be involved in the endothelial-inhibitory action of NK4. To address this issue, we tested the effect of neutralizing antibody on the growth and migration of endothelial cells (Fig. 4*D*). In contrast to NK4, anti-HGF antibody did not inhibit bFGF- and VEGF-induced endothelial growth. On the other hand, the stimulatory effect of HGF on endothelial cell growth was almost completely inhibited by anti-HGF antibody, equally to NK4. Similarly, endothelial cell migration mediated by bFGF and VEGF was not affected by anti-HGF antibody (not shown).

Effects of NK4 on Receptor Tyrosine Phosphorylation and ERK1/2 Activation. To investigate the possibility that the binding of NK4 to the Met receptor may modify ligand-receptor interaction and subsequent activation of signaling events, or that NK4 itself may interfere with ligand-dependent activation of the receptor for angiogenic growth factors, we examined the tyrosine phosphorylation state of c-Met in endothelial cells. HGF, but not bFGF or VEGF, specifically activated c-Met in endothelial cells by increasing the phosphorylation on tyrosine residues, as shown by immunoprecipitation and Western blotting (Fig. 5*A*). NK4 inhibited HGF-induced phosphorylation of the Met receptor. On the other hand, NK4 alone, in concentrations up to 1000 nM (67 μg/ml), did not induce tyrosine phosphorylation of c-Met, and NK4 (300 nM) also failed to activate c-Met in the presence of bFGF or VEGF.

Next, to assess whether NK4 modifies the activation of the VEGF receptor, we measured the phosphorylation state of KDR, the activation of which is largely responsible for VEGF-mediated endothelial growth and migration. VEGF, but not HGF or NK4, stimulated tyrosine phosphorylation of KDR (Fig. 5*B*). In contrast with the blockade of HGF-Met coupling by NK4, the VEGF-induced phosphorylation of KDR was not inhibited by NK4. Because activation of ERK1/2 (p44/42 MAPK) is closely involved in the process of angiogenesis induced by various angiogenic stimuli, we next analyzed activation of ERK1/2 (Fig. 5*C*). HGF, bFGF, and VEGF induced prominent activation of ERK1/2, whereas NK4 did not inhibit the bFGF- or VEGF-induced activation of ERK1/2 but did inhibit HGF-induced ERK1/2 activation. Therefore, NK4 does not inhibit early intracellular signaling events driven by bFGF and VEGF, yet NK4 inhibits bFGF- and VEGF-induced angiogenic responses in endothelial cells.

***In-Vivo* Antiangiogenic Activity of NK4.** To study the angiostatic activity of NK4 *in vivo*, NK4 was tested using CAM (Fig. 6*A*). In controls, CAMs with avascular zones were never found. However, NK4 inhibited new blood vessel formation in a dose-dependent manner as determined by the formation of avascular zones (Fig. 6*A*); at a dose of 60 μg NK4/disk, avascular zones were seen in 61.5% of embryos ($P < 0.01$). Importantly, the inhibitory activity of NK4 on angiogenesis in chick CAMs diminished when NK4 was tested after heat-treatment. BSA (60 μg/disk) had no inhibitory effect on angiogenesis in the CAM assay. The results indicate that NK4 has antiangiogenic activity *in vivo* as well as *in vitro*.

To further confirm the antiangiogenic activity of NK4 *in vivo*, the effects of NK4 on bFGF-induced neovascularization were tested in the rabbit cornea assay (Fig. 6*B*). Application of 100 ng of bFGF induced neovascularization in 70% of the corneas (7 of 10), whereas

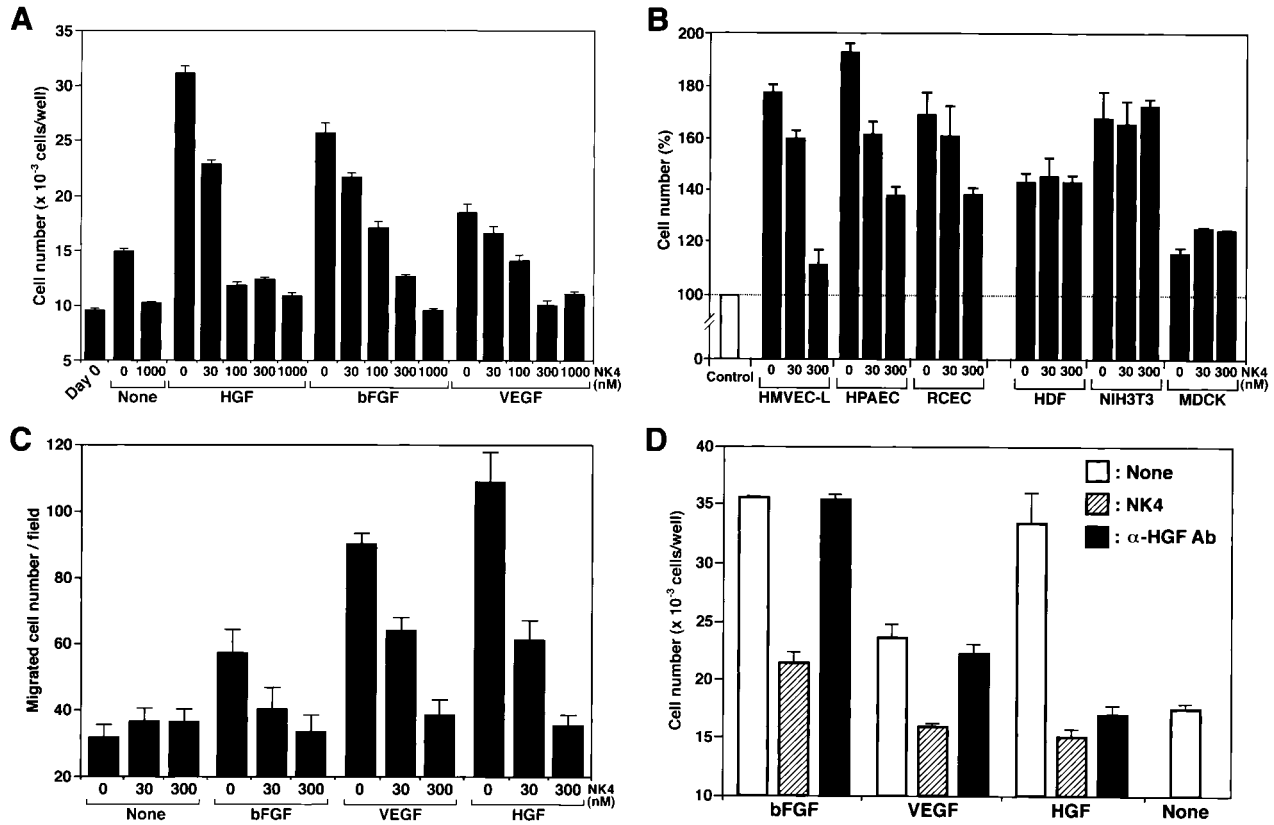

Fig. 4. *A*, inhibition of HMVEC proliferation by NK4. The cells were cultured with 3 ng/ml bFGF, 10 ng/ml VEGF, or 10 ng/ml HGF with or without various amounts of NK4 for 72 h. *B*, effects of NK4 on proliferation of various endothelial cells and nonendothelial cells. Cells were cultured in the presence of 3 ng/ml bFGF, with or without various amounts of NK4 for 72 h. *HMVEC-L*, human lung-derived microvascular endothelial cells; *HPAEC*, human pulmonary artery endothelial cells; RCEC, rat coronary endothelial cells; *HDF*, human dermal fibroblasts; *NIH3T3*, mouse fibroblasts; *MDCK*, canine renal epithelial cells. *C*, inhibition of HMVEC migration by NK4. The cells were cultured with 3 ng/ml bFGF, 3 ng/ml VEGF, or 10 ng/ml HGF with or without NK4 for 5 h. *D*, distinct effects of anti-HGF antibody (*α-HGF Ab*) and NK4 on HMVEC growth. The cells were cultured in the absence or presence of 3 ng/ml bFGF, 10 ng/ml VEGF, or 3 ng/ml HGF with or without 300 nM NK4 or 10 μg/ml anti-HGF antibody for 72 h. Each value represents the mean ± SE.

simultaneous application of NK4 dose-dependently inhibited bFGF-induced neovascularization. NK4 at 1000 ng significantly inhibited bFGF-induced neovascularization to 22% ($P < 0.05$). Heat-treated NK4 had no effect on bFGF-induced neovascularization, and NK4 alone had no effect on neovascularization in the cornea. Application of HGF in the cornea assay induced neovascularization and NK4 had inhibitory effects on HGF-induced neovascularization (not shown).

DISCUSSION

Originally we prepared NK4 as a competitive receptor-antagonist devoid of its own HGF-related activities. NK4 inhibits biological activities of HGF, including mitogenic, motogenic, and morphogenic activities (17, 18). Together with our present finding that NK4 functions as a specific angiogenesis inhibitor, NK4 is bifunctional: it acts as an HGF-antagonist and also as an angiogenesis inhibitor. These distinct functions seem to occur through distinct mechanisms, because the inhibitory effects of NK4 on angiogenesis induced by angiogenic factors other than HGF are unlikely to be mediated via its potential to competitively block coupling between HGF and its receptor, c-Met. In fact, the blockade of coupling of HGF and the c-Met receptor by an anti-HGF antibody did not result in inhibition of endothelial proliferation and migration stimulated by other angiogenic growth factors.

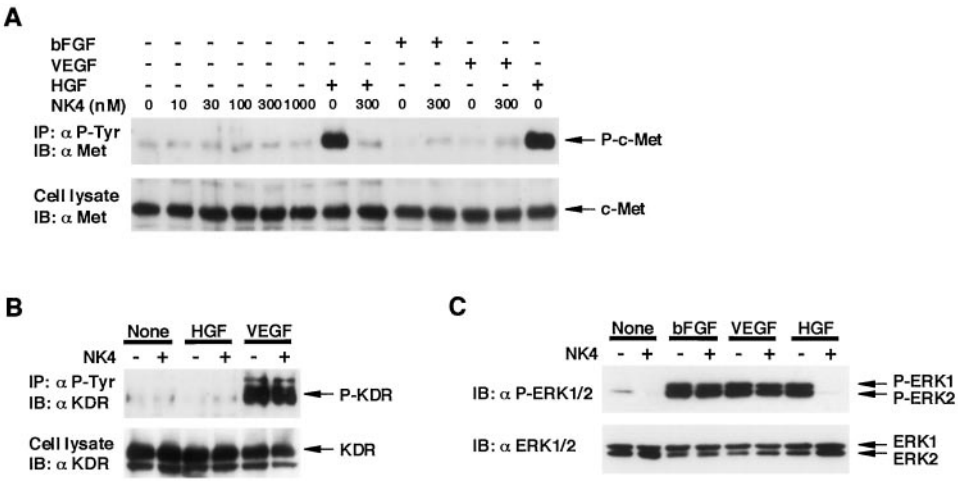

Fig. 5. Effects of NK4 on receptor tyrosine phosphorylation and ERK1/2 (p44/42 MAPK) activation. *A*, effects of NK4 on activation of c-Met. HMVECs were treated with NK4 alone or in combination with HGF, bFGF, or VEGF (10 ng/ml each) for 10 min. Cell lysates were immunoprecipitated with anti-phosphotyrosine antibody and blotted with anti-c-Met antibody. *B*, effects of NK4, HGF, and VEGF on activation of KDR/VEGF receptor. HMVECs were treated with NK4 (300 nM) alone or in combination with VEGF or HGF (10 ng/ml each) for 10 min. Tyrosine phosphorylated KDR was detected as described above. *C*, effects of NK4 on activation of ERK1/2 (p44/42 MAPK). HMVECs were treated with NK4 (300 nM) alone or in combination with bFGF, VEGF, or HGF (10 ng/ml each) for 10 min. Activation of ERK1/2 was assayed by Western blots of cell lysates with antibodies against phosphorylated ERK1/2 (*top*) or total ERK as a loading control (*bottom*).

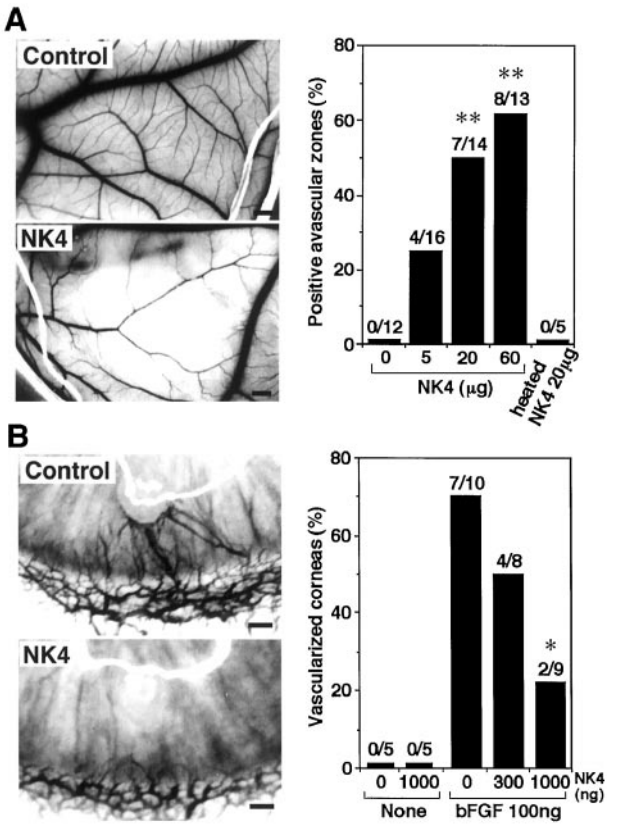

Fig. 6. *A*, inhibitory effect of NK4 on angiogenesis in chick CAMs. Representative appearance of chick CAMs implanted with a disk containing saline or 20 μg of NK4 (*left*). For each CAM, a methylcellulose disk containing sample was removed and photographed. *Bars*, 1 mm. Dose-dependent inhibition of angiogenesis in CAMs by NK4 (**, $P < 0.01$; *right*). The number of CAMs with avascular zones over the total number of CAMs is indicated *above* each column. *B*, suppression of rabbit corneal neovascularization by NK4. Appearance of neovascularization in cornea (*left*). A pellet containing bFGF 100 ng or bFGF 100 ng plus NK4 1000 ng/pellet was surgically implanted in rabbit cornea. *Bars*, 1 mm. Dose-dependent suppression of bFGF-induced neovascularization by NK4 (*, $P < 0.05$; *right*). The number of vascularized corneas over the total number of corneas is indicated *above* each column.

Neither bFGF- nor VEGF-induced angiogenic response was mediated by the activation of c-Met in endothelial cells. In addition, bFGF- or VEGF-induced angiogenic signals in the early phase are not blocked by NK4, because NK4 allowed VEGF-induced KDR/VEGF receptor tyrosine phosphorylation and bFGF- or VEGF-induced ERK1/2 activation. Thus, a likely explanation is that NK4 may exert angiostatic signals through putative binding molecules on endothelial cells, although we cannot rule out the possibility that the c-Met receptor may participate in bFGF/VEGF-mediated angiogenesis through unknown mechanisms.

With regard to the structure and function of NK4, it is noteworthy that NK4 has a significant structural similarity with angiostatin, a potent angiogenesis inhibitor (29). Angiostatin is an internal fragment of plasminogen that encompasses the first four kringle domains, and the amino acids sequence homology between four kringles of NK4 and angiostatin reaches 47%. Previous studies showed that individual kringle domains of angiostatin, the fifth kringle domain of plasminogen, and the prothrombin kringle-2 domain have antiangiogenic activity (44–46). Our preliminary results indicated that antiangiogenic activity of NK4 seems to reside within kringle domains.[4] These results suggest the possibility that a structural motif conserved in some kringle domains may be involved in inhibiting angiogenesis. However, it is equally probable that NK4 inhibits angiogenesis through a mechanism distinct from angiostatin, because NK4 inhibits DNA synthesis and induces cell cycle arrest,[5] whereas angiostatin increases endothelial apoptosis without inhibiting DNA synthesis (34, 47).

Of particular importance in the present study is the *in vivo* inhibition of tumor metastasis by NK4. The metastatic cascade is composed of multiple steps: (*a*) induction of angiogenesis; (*b*) dissociation of tumor cells; (*c*) invasion through the extracellular matrix; (*d*) intravasation; (*e*) transport in the circulation; (*f*) arrest in a distant capillary bed, and (*g*) extravasation followed by the establishment of secondary foci (48). In our experimental model, inhibition of lung metastasis might be the result of suppression of early events (*i.e.*, angiogenesis and invasion) in the primary tumors, because NK4-treatment was terminated 10 days before evaluation of lung metastasis, and this no-treatment period might have allowed for the growth of secondary tumors (Fig. 2*A*). Considering the bifunctionality of NK4 (HGF antagonist and angiogenesis inhibitor), we can raise the possibility that NK4 may have suppressed lung metastasis by both actions: one, the inhibition of tumor angiogenesis, and the other, the blockade of HGF-mediated dissociation and invasion of tumor cells. There are several reports that aggressive angiogenesis in primary tumors correlates well with the high frequency of distant metastasis in cancer patients (49, 50). Reduced angiogenesis by NK4 in the primary tumors may have decreased the incidence of intravasation of tumor cells, the result being inhibition of lung metastasis. On the other hand, HGF potently stimulates dissociation, migration, and invasion of tumor cells (12, 51–53), and the induction of extracellular protease networks (such as urokinase-type plasminogen activator and a variety of matrix metalloproteases) by HGF is involved in invasion and subsequent metastasis (18, 53, 54). Indeed, HGF potently stimulated *in-vitro* invasion of LLC and Jyg-MC cells through Matrigel (which mimics basement membrane), whereas NK4 blocked the HGF-induced invasion of these tumor cells (Fig. 1*B*). Thus, the blockade of HGF-Met coupling by NK4 might have suppressed the invasion, motility, and subsequent intravasation of tumor cells, leading to the inhibition of lung metastasis, in our model.

Cutting off the vascular supply for malignant tumors by an angiogenesis inhibitor is expected to be a promising form of cancer therapy. On the other hand, an angiogenesis inhibitor will not modify the invasive and metastatic phenotype of cancer cells, though angiogenesis-dependent tumor invasion is suppressed by halting angiogenesis in some cases (55). A number of studies indicated a critical role for HGF in the invasion and metastasis of a wide variety of malignant tumor cells (6–10), and inhibition of HGF-Met coupling or signal transduction from the Met has been implicated as a therapeutic strategy to prevent cancer invasion and metastasis (18, 56–58). On the basis of the bifunctional characteristics of NK4 to target both tumor angiogenesis and HGF-mediated invasion, the possibility that NK4 can function as a therapeutic for subjects with cancer warrants ongoing studies.

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REFERENCES

1. Nakamura, T., Nawa, K., and Ichihara, A. Partial purification and characterization of hepatocyte growth factor from serum of hepatectomized rats. Biochem. Biophys. Res. Commun., *122:* 1450–1459, 1984.

[4] Unpublished results.

[5] Our unpublished data.

2. Nakamura, T., Nishizawa, T., Hagiya, M., Seki, T., Shimonishi, M., Sugimura, A., Tashiro, K., and Shimizu, S. Molecular cloning and expression of human hepatocyte growth factor. Nature (Lond.), *342:* 440–443, 1989.
3. Zarnegar, R., and Michalopoulos, G. K. The many faces of hepatocyte growth factor: from hepatopoiesis to hematopoiesis. J. Cell Biol., *129:* 1177–1180, 1995.
4. Matsumoto, K., and Nakamura, T. Hepatocyte growth factor (HGF) as a tissue organizer for organogenesis and regeneration. Biochem. Biophys. Res. Commun., *239:* 639–644, 1997.
5. Birchmeier, C., and Gherardi, E. Developmental roles of HGF/SF and its receptor, the c-Met tyrosine kinase. Trends Cell Biol., *8:* 404–410, 1998.
6. Cortner, J., Vande Woude, G. F., and Rong, S. The Met-HGF/SF autocrine signaling mechanism is involved in sarcomagenesis. EXS, *74:* 89–121, 1995.
7. Rosen, E. M., and Goldberg, I. D. Scatter factor and angiogenesis. Adv. Cancer Res., *67:* 257–279, 1995.
8. Jeffers, M., Rong, S., and Vande Woude, G. F. Hepatocyte growth factor/scatter factor-Met signaling in tumorigenicity and invasion/metastasis. J. Mol. Med., *74:* 505–513, 1996.
9. To, C. T., and Tsao, M. S. The roles of hepatocyte growth factor/scatter factor and met receptor in human cancers. Oncol. Rep., *5:* 1013–1024, 1998.
10. Jiang, W. G., Hiscox, S., Matsumoto, K., and Nakamura, T. Hepatocyte growth factor/scatter factor, its molecular, cellular and clinical implications in cancer. Crit. Rev. Oncol. Hematol., *29:* 209–248, 1999.
11. Seslar, S. P., Nakamura, T., and Byers, S. W. Regulation of fibroblast hepatocyte growth factor/scatter factor expression by human breast carcinoma cell lines and peptide growth factors. Cancer Res., *53:* 1233–1238, 1993.
12. Nakamura, T., Matsumoto, K., Kiritoshi, A., Tano, Y., and Nakamura, T. Induction of hepatocyte growth factor in fibroblasts by tumor-derived factors affects invasive growth of tumor cells: *in vitro* analysis of tumor-stromal interactions. Cancer Res., *57:* 3305–3313, 1997.
13. Rong, S., Jeffers, M., Resau, J. H., Tsarfaty, I., Oskarsson, M., and Vande Woude, G. F. Met expression and sarcoma tumorigenicity. Cancer Res., *53:* 5355–5360, 1993.
14. Schmidt, L., Duh, F. M., Chen, F., Kishida, T., Glenn, G., Choyke, P., Scherer, S. W., Zhuang, Z., Lubensky, I., Dean, M., Allikmets, R., Chidambaram, A., Bergerheim, U. R., Feltis, J. T., Casadevall, C., Zamarron, A., Bernues, M., Richard, S., Lips, C. J., Walther, M. M., Tsui, L. C., Geil, L., Orcutt, M. L., Stackhouse, T., and Zbar, B. Germline and somatic mutations in the tyrosine kinase domain of the MET proto-oncogene in papillary renal carcinomas. Nat. Genet., *16:* 68–73, 1997.
15. Jeffers, M., Schmidt, L., Nakaigawa, N., Webb, C. P., Weirich, G., Kishida, T., Zbar, B., and Vande Woude, G. F. Activating mutations for the met tyrosine kinase receptor in human cancer. Proc. Natl. Acad. Sci. USA, *94:* 11445–11450, 1997.
16. Michieli, P., Basilico, C., Pennacchietti, S., Maffe, A., Tamagnone, L., Giordano, S., Bardelli, A., and Comoglio, P. M. Mutant Met-mediated transformation is ligand-dependent and can be inhibited by HGF antagonists. Oncogene, *18:* 5221–5231, 1999.
17. Date, K., Matsumoto, K., Shimura, H., Tanaka, M., and Nakamura, T. HGF/NK4 is a specific antagonist for pleiotrophic actions of hepatocyte growth factor. FEBS Lett., *420:* 1–6, 1997.
18. Date, K., Matsumoto, K., Kuba, K., Shimura, H., Tanaka, M., and Nakamura, T. Inhibition of tumor growth and invasion by a four-kringle antagonist (HGF/NK4) for hepatocyte growth factor. Oncogene, *17:* 3045–3054, 1998.
19. Chan, A. M., Rubin, J. S., Bottaro, D. P., Hirschfield, D. W., Chedid, M., and Aaronson, S. A. Identification of a competitive HGF antagonist encoded by an alternative transcript. Science (Washington DC), *254:* 1382–1385, 1991.
20. Silvagno, F., Follenzi, A., Arese, M., Prat, M., Giraudo, E., Gaudino, G., Camussi, G., Comoglio, P. M., and Bussolino, F. *In vivo* activation of met tyrosine kinase by heterodimeric hepatocyte growth factor molecule promotes angiogenesis. Arterioscler. Thromb. Vasc. Biol., *15:* 1857–1865, 1995.
21. Cioce, V., Csaky, K. G., Chan, A. M. L., Bottaro, D. P., Taylor, W. G., Jensen, R., Aaronson, S. A., and Rubin, J. S. Hepatocyte growth factor (HGF)/NK1 is a naturally occurring HGF/scatter factor variant with partial agonist/antagonist activity. J. Biol. Chem., *271:* 13110–13115, 1996.
22. Jakubczak, J. L., LaRochelle, W. J., and Merlino, G. NK1, a natural splice variant of hepatocyte growth factor/scatter factor, is a partial agonist *in vivo*. Mol. Cell. Biol., *18:* 1275–1283, 1998.
23. Folkman, J. Seminars in Medicine of the Beth Israel Hospital, Boston. Clinical applications of research on angiogenesis, N. Engl. J. Med., *333:* 1757–1763, 1995.
24. Risau, W. Mechanisms of angiogenesis. Nature (Lond.), *386:* 671–674, 1997.
25. Hanahan, D., and Folkman, J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. Cell, *86:* 353–364, 1996.
26. Bouck, N. Tumor angiogenesis: the role of oncogenes and tumor suppressor genes. Cancer Cells, *2:* 179–185, 1990.
27. Maione, T. E., Gray, G. S., Petro, J., Hunt, A. J., Donner, A. L., Bauer, S. I., Carson, H. F., and Sharpe, R. J. Inhibition of angiogenesis by recombinant human platelet factor-4 and related peptides. Science (Washington DC), *247:* 77–79, 1990.
28. Brooks, P. C., Clark, R. A., and Cheresh, D. A. Requirement of vascular integrin $\alpha v\beta 3$ for angiogenesis. Science (Washington DC), *264:* 569–571, 1994.
29. O'Reilly, M. S., Holmgren, L., Shing, Y., Chen, C., Rosenthal, R. A., Moses, M., Lane, W. S., Cao, Y., Sage, E. H., and Folkman, J. Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. Cell, *79:* 315–328, 1994.
30. O'Reilly, M. S., Boehm, T., Shing, Y., Fukai, N., Vasios, G., Lane, W. S., Flynn, E., Birkhead, J. R., Olsen, B. R., and Folkman, J. Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. Cell, *88:* 277–285, 1997.
31. Ingber, D., Fujita, T., Kishimoto, S., Sudo, K., Kanamaru, T., Brem, H., and Folkman, J. Synthetic analogues of fumagillin that inhibit angiogenesis and suppress tumour growth. Nature (Lond.), *348:* 555–557, 1990.
32. D'Amato, R. J., Loughnan, M. S., Flynn, E., and Folkman, J. Thalidomide is an inhibitor of angiogenesis. Proc. Natl. Acad. Sci. USA, *91:* 4082–4085, 1994.
33. Seki, T., Ihara, I., Sugimura, A., Shimonishi, M., Nishizawa, T., Asami, O., Hagiya, M., Nakamura, T., and Shimizu, S. Isolation and expression of cDNA for different forms of hepatocyte growth factor from human leukocytes. Biochem. Biophys. Res. Commun., *172:* 321–327, 1990.
34. Lucas, R., Holmgren, L., Garcia, I., Jimenez, B., Mandriota, S. J., Borlat, F., Sim, B. K., Wu, Z., Grau, G. E., Shing, Y., Soff, G. A., Bouck, N., and Pepper, M. S. Multiple forms of angiostatin induce apoptosis in endothelial cells. Blood, *92:* 4730–4741, 1998.
35. Matsumiya, G., Shirakura, R., Miyagawa, S., Izutani, H., Sawa, Y., Nakata, S., and Matsuda, H. Analysis of rejection mechanism in the rat to mouse cardiac xenotransplantation. Role and characteristics of anti-endothelial cell antibodies. Transplantation, *57:* 1653–1660, 1994.
36. Soker, S., Takashima, S., Miao, H. Q., Neufeld, G., and Klagsbrun, M. Neuropilin-1 is expressed by endothelial and tumor cells as an isoform-specific receptor for vascular endothelial growth factor. Cell, *92:* 735–745, 1998.
37. Holmgren, L., O'Reilly, M. S., and Folkman, J. Dormancy of micrometastases: balanced proliferation and apoptosis in the presence of angiogenesis suppression. Nat. Med., *1:* 149–153, 1995.
38. Tanaka, N. G., Sakamoto, N., Tohgo, A., Nishiyama, Y., and Ogawa, H. Inhibitory effects of anti-angiogenic agents on neovascularization and growth of the chorioallantoic membrane (CAM). The possibility of a new CAM assay for angiogenesis inhibition. Exp. Pathol., *30:* 143–150, 1986.
39. Gimbrone, M. A., Jr., Cotran, R. S., Leapman, S. B., and Folkman, J. Tumor growth and neovascularization: an experimental model using the rabbit cornea. J. Natl. Cancer Inst., *52:* 413–427, 1974.
40. Polverini, P. J., Bouck, N. P., and Rastinejad, F. Assay and purification of naturally occurring inhibitor of angiogenesis. Methods Enzymol., *198:* 440–450, 1991.
41. Klintworth, G. K. Corneal Angiogenesis: A Comprehensive Review. New York: Springer-Verlag New York, Inc., 1991.
42. O'Reilly, M. S., Holmgren, L., Chen, C., and Folkman, J. Angiostatin induces and sustains dormancy of human primary tumors in mice. Nat. Med., *2:* 689–692, 1996.
43. Jiang, W. G., Hiscox, S. E., Parr, C., Martin, T. A., Matsumoto, K., Nakamura, T., and Mansel, R. E. Antagonistic effect of NK4, a novel hepatocyte growth factor variant, on *in vitro* angiogenesis of human vascular endothelial cells. Clin. Cancer Res., *5:* 3695–3703, 1999.
44. Cao, Y., Ji, R. W., Davidson, D., Schaller, J., Marti, D., Sohndel, S., McCance, S. G., O'Reilly, M. S., Llinas, M., and Folkman, J. Kringle domains of human angiostatin. Characterization of the anti-proliferative activity on endothelial cells. J. Biol. Chem., *271:* 29461–29467, 1996.
45. Cao, Y., Chen, A., An, S. S. A., Ji, R. W., Davidson, D., and Llinas, M. Kringle 5 of plasminogen is a novel inhibitor of endothelial cell growth. J. Biol. Chem., *272:* 22924–22928, 1997.
46. Lee, T. H., Rhim, T., and Kim, S. S. Prothrombin kringle-2 domain has a growth inhibitory activity against basic fibroblast growth factor-stimulated capillary endothelial cells. J. Biol. Chem., *273:* 28805–28812, 1998.
47. Claesson-Welsh, L., Welsh, M., Ito, N., Anand Apte, B., Soker, S., Zetter, B., O'Reilly, M., and Folkman, J. Angiostatin induces endothelial cell apoptosis and activation of focal adhesion kinase independently of the integrin-binding motif RGD, Proc. Natl. Acad. Sci. USA, *95:* 5579–5583, 1998.
48. Fidler, I. J. Critical factors in the biology of human cancer metastasis: twenty-eighth G. H. A. Clowes memorial award lecture. Cancer Res., *50:* 6130–6138, 1990.
49. Liotta, L. A., Kleinerman, J., and Saidel, G. M. Quantitative relationships of intravascular tumor cells, tumor vessels, and pulmonary metastases following tumor implantation. Cancer Res., *34:* 997–1004, 1974.
50. Weidner, N. Intratumor microvessel density as a prognostic factor in cancer. Am. J. Pathol., *147:* 9–19, 1995.
51. Weidner, K. M., Behrens, J., Vandekerckhove, J., and Birchmeier, W. Scatter factor: molecular characteristics and effect on the invasiveness of epithelial cells. J. Cell Biol., *111:* 2097–2108, 1990.
52. Matsumoto, K., Matsumoto, K., Nakamura, T., and Kramer, R. H. Hepatocyte growth factor/scatter factor induces tyrosine phosphorylation of focal adhesion kinase (p125FAK) and promotes migration and invasion by oral squamous cell carcinoma cells. J. Biol. Chem., *269:* 31807–31813, 1994.
53. Jeffers, M., Rong, S., and Vande Woude, G. F. Enhanced tumorigenicity and invasion-metastasis by hepatocyte growth factor/scatter factor-met signalling in human cells concomitant with induction of the urokinase proteolysis network. Mol. Cell. Biol., *16:* 1115–1125, 1996.
54. Pepper, M. S., Matsumoto, K., Nakamura, T., Orci, L., and Montesano, R. Hepatocyte growth factor increases urokinase-type plasminogen activator (u-PA) and u-PA receptor expression in Madin-Darby canine kidney epithelial cells. J. Biol. Chem., *267:* 20493–20496, 1992.
55. Skobe, M., Rockwell, P., Goldstein, N., Vosseler, S., and Fusenig, N. E. Halting angiogenesis suppresses carcinoma cell invasion. Nat. Med., *3:* 1222–1227, 1997.
56. Abounader, R., Ranganathan, S., Lal, B., Fielding, K., Book, A., Dietz, H., Burger, P., and Laterra, J. Reversion of human glioblastoma malignancy by U1 small nuclear RNA/ribozyme targeting of scatter factor/hepatocyte growth factor and c-met expression. J. Natl. Cancer Inst., *91:* 1548–1556, 1999.
57. Bardelli, A., Longati, P., Williams, T. A., Benvenuti, S., and Comoglio, P. M. A peptide representing the carboxyl-terminal tail of the met receptor inhibits kinase activity and invasive growth. J. Biol. Chem., *274:* 29274–29281, 1999.
58. Webb, C. P., Hose, C. D., Koochekpour, S., Jeffers, M., Oskarsson, M., Sausville, E., Monks, A., and Vande Woude, G. F. The geldanamycins are potent inhibitors of the hepatocyte growth factor/scatter factor-met-urokinase plasminogen activator-plasmin proteolytic network. Cancer Res., *60:* 342–349, 2000.

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