Reduced HGF expression in subcutaneous CT26 tumor genetically modified to secrete NK4 and its possible relation with antitumor effects

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Tumor-stromal interactions, which are regulated by stromal-derived HGF and tumor-derived HGF inducers, are essential for tumor cell acquisition of such malignant properties as invasion and metastasis. NK4, a proteolytic cleavage product of HGF, has antitumor activities as both an HGF antagonist and an angiogenesis inhibitor. In this study, we examined the *in vitro* and *in vivo* behaviors of mouse colon adenocarcinoma CT26 cells modified by gene transfer to secrete NK4, and investigated the influence of NK4 on expression of HGF and HGF inducers associated with tumor-stromal interactions. *In vitro* cell proliferation rates of NK4 transfectant (CT26-NK4) and mock transfectant (CT26-NEO) were essentially the same, and scattering and invasion were stimulated by HGF in CT26-NEO, but not in CT26-NK4. In syngeneic BALB/c female mice, subcutaneous tumor growth of CT26-NK4 was potently suppressed, and the survival was prolonged significantly. Immunohistochemistry showed significantly decreased microvessels and increased apoptotic cells in CT26-NK4 tumor compared with control. Interestingly, HGF, strongly expressed in CT26-NEO tumor stroma, was reduced in CT26-NK4. *In vitro*, conditioned medium of CT26-NK4 inhibited fibroblast-derived HGF production, which was increased by that of CT26-NEO. Moreover, although similar constitutive expression levels of PDGF and TGF-α (both HGF inducers) were detected in CT26-NK4 and CT26-NEO in semiquantitative RT-PCR analyses, the expression was up-regulated by HGF in CT26-NEO, but not CT26-NK4. These results suggest that NK4 may exert antitumor activities not only by antagonizing HGF, but also by inhibiting HGF amplification via tumor-stromal interactions. Continuous, abundant NK4 production induced at a tumor site by gene transfer should show multiple antitumor activities with potential therapeutic benefit. (Cancer Sci 2004; 95: 321–327)

Tumor-stromal interactions are essential for tumor cell acquisition of invasive and metastatic potential. Previous studies demonstrated that HGF is a predominantly stromal-derived factor involved in invasive and metastatic tumor-stromal interactions.[1–5] Importantly, a variety of cancer cells produce inducers of stromal HGF production, including interleukin (IL)-1α, IL-1β, basic fibroblast growth factor (bFGF), TGF-α, PDGF, and prostaglandin (PG) E2.[1–5] Thus, there is a mutual interaction between cancer cells and stromal cells mediated by HGF and HGF inducers.

HGF, originally identified and cloned as a potent mitogen for hepatocytes, is composed of an α chain that contains a NH_2-terminal hairpin and four kringle domains, and the catalytically inactive, serine protease-like β chain.[6,7] HGF facilitates tumor cell motility by binding to the c-Met receptor expressed on various malignant cells including colorectal,[8] gastric,[9] esophageal,[10] breast,[11] hepatocellular,[12,13] and lung[14,15] cancers. HGF also binds to the receptor expressed on endothelial cells that stimulates angiogenesis,[16,17] a process critical to continued growth of the primary tumor[18,19] and a factor that increases the opportunity for hematogenous metastasis of tumor cells. Previous studies have shown that increased vascularity is inversely correlated with prognosis in cancers originating in breast,[20] lung,[21] prostate,[22] uterine cervix,[23] stomach,[24] and colon.[25]

NK4, a product of proteolytic digestion of HGF, has been identified as a potent HGF antagonist. It includes the NH_2-terminal hairpin domain and four kringle domains of the HGFα subunit.[26] NK4 binding to the c-Met receptor competitively antagonizes HGF-induced tyrosine phosphorylation of the receptor,[27] resulting in complete inhibition of biological events driven by HGF/c-Met receptor signaling; these include invasion and metastasis by various tumor cells including those from colon,[27] pancreatic,[28] gallbladder,[29] lung,[30] and prostate[31] cancers. Furthermore, a recent study demonstrated that NK4 exerts a potent inhibitory effect not only on HGF/c-Met-dependent angiogenesis, but also on the HGF/c-Met-independent angiogenesis induced by bFGF and vascular endothelial growth factor (VEGF).[30] These bifunctional properties of NK4 as an HGF antagonist and an angiogenesis inhibitor suggest therapeutic promise against cancer.

On the other hand, although NK4 is well demonstrated to inhibit HGF-induced tumor cell acquisition of malignant phenotypes by antagonizing HGF in *in vitro* experiments, the influence of NK4 on *in vivo* expression of stromal-derived HGF and tumor-derived HGF inducers, remains to be elucidated in detail.

In the present study, we transfected the *NK4* gene into mouse colon adenocarcinoma CT26 cells, which show a highly malignant phenotype *in vivo*, and established a stable transfectant producing abundant NK4 (CT26-NK4). Then, we investigated the *in vitro* behaviors of CT26-NK4 and its *in vivo* antitumor effects using a subcutaneous (s.c.) tumor model in syngeneic BALB/c mice. Furthermore, we analyzed the antitumor mechanisms with special reference to the actions of NK4 on *in vivo* tumor-stromal interactions.

Materials and Methods

Reagents. Human recombinant HGF was purified from the conditioned medium of Chinese hamster ovary cells transfected with human HGF cDNA.[6,32] Polyclonal anti-human HGF antibody was purchased from R&D Systems (Minneapolis, MN). According to the manufacturer's notification, 5 μg/ml of this anti-HGF antibody completely neutralized the biological activities of 10 ng/ml HGF.

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Abbreviations: HGF, hepatocyte growth factor; PDGF, platelet-derived growth factor; TGF, transforming growth factor; RT-PCR, reverse transcription-polymerase chain reaction.

Mice. BALB/c female mice, 8 to 10 weeks old, purchased from Shimizu Laboratory Animal Center (Kyoto) were maintained free of specific pathogens. The investigation protocols were approved by the Ethics Committee of Kyoto Prefectural University of Medicine.

Cell and culture. CT26 is an undifferentiated colon adenocarcinoma cell line originally derived from a cancer produced by intrarectal injections of *N*-nitroso-*N*-methylurethamine in a female BALB/c mouse.[33] Cells were maintained in RPMI 1640 (Nacalai Tesque, Kyoto) supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin (Sigma, St. Louis, MO), and 10% heat-inactivated fetal bovine serum (FBS; JRH Bioscience, Lenexa, KS).

Plasmids and transfection with NK4 cDNA. The human NK4 expression plasmid, pcDNA3/NK4 is a pcDNA3-derived plasmid containing the human NK4 sequence under the control of the cytomegalovirus promoter/enhancer.[26] CT26 cells were transfected with pcDNA3/NK4 by electroporation. Neomycin-resistant cells then were selected in 800 μg/ml G418 (Sigma) for 14 days starting at 3 days after transfection. Selected transfectants were cloned by the limiting dilution method. NK4 secretion into the supernatant from each clone was determined by a human HGF enzyme-linked immunosorbent assay (ELISA; Institute of Immunology, Tokyo). Murine HGF production by the transfectants and parental CT26 cells was confirmed by a rat HGF ELISA (Institute of Immunology). A transfectant expressing a large amount of NK4 was designated CT26-NK4. Cells transfected with the neomycin-resistance gene (pSVneo) alone were used as a control (CT26-NEO). CT26-NK4 and CT26-NEO expressed almost the same levels of c-Met receptor as the parental cells, and HGF-stimulated tyrosine phosphorylation of the receptor in CT26-NEO was strongly inhibited in CT26-NK4 (data not shown).

To confirm NK4 production by parental cells or the transfectants, cells were plated at 10^6 cells/well onto 10 cm culture dishes and cultured for 24 h. The media were collected for NK4 determination with the human HGF ELISA.

Assay for cell proliferation, invasion, and colony scattering. For cell proliferation assay, each transfectant in RPMI 1640 with 10% FBS was plated at 2.5×10^3 cells/well onto 24-well tissue culture plates and cultured for 24 h. Culture media then were replaced with fresh RPMI 1640 with 5% FBS, and the cells were cultured in the absence or presence of 10 ng/ml HGF with or without 5 μg/ml anti-HGF antibody. After 1, 3, and 5 days, the cells were counted using a hemocytometer.

Invasion by transfectants was examined *in vitro* using a 24-well Matrigel invasion chamber (Becton Dickinson, Bedford, MA) equipped with a filter membrane with 8 μm pores. Cells were plated at 1×10^4 cells/well in RPMI with 10% FBS onto the inner compartment of the chamber. The cells were cultured in the absence or presence of 10 ng/ml HGF with or without 5 μg/ml anti-HGF antibody for 24 h, fixed in 70% ethanol, and counterstained with hematoxylin. Cells invading the opposite side of the membrane through the pores were counted in five randomly selected microscopic fields (×200). Proliferation and invasion assays were performed in triplicate.

For the colony scattering assay, each transfectant was plated at 250 cells/cm^2 in RPMI with 10% FBS onto 6-well culture plates and cultured for 4–7 days. The media were replaced with fresh RPMI containing 2% FBS, then 10 ng/ml HGF was added to each well, and scattering of the cells was microscopically observed after incubation for 15 h.

Measurement of NK4 in tumors transplanted *in vivo*. In a preliminary animal experiment, we determined tissue NK4 in transplanted tumors by ELISA. After s.c. inoculation of CT26-NK4 or CT26-NEO cells into BALB/c mice (see below), the tumors were removed at 7 or 14 days. Each tumor was homogenized in 4 vol./g tissue of lysis buffer composed of 10 m*M* Tris-HCl (pH 7.5), 2 *M* NaCl, 1 m*M* phenylmethylsulfonyl fluoride, 1 m*M* EDTA, and 0.01% Tween 80. Homogenates were centrifuged at 105,000*g* for 1 h, and the supernatants were used as a crude tissue extract. To evaluate systemic NK4, extracts from liver, kidney, lung, and blood obtained 7 or 14 days after inoculation of each transfectant also were prepared and assayed as described above.

Animal experiment on NK4 antitumor effects. To examine the biological activity of NK4 *in vivo*, 5×10^5 cells of CT26-NK4 or CT26-NEO in 0.1 ml of PBS were inoculated s.c. into syngeneic BALB/c mice in the right lower flank (n=10 for each group). Two perpendicular diameters of the resulting tumors were measured every 3 to 4 days using a caliper. Tumor volumes were calculated in mm^3 as $0.52\times(\text{width})^2\times(\text{length})$.

Survival was analyzed by the Kaplan-Meier method and compared between groups by use of the log-rank test.

Histological analyses. Tumors were removed at 7 days after s.c. inoculation of CT26-NK4 or CT26-NEO, fixed in 10% formalin or 70% ethanol for 1 day, and embedded in paraffin.

For staining of microvessels, tissue sections fixed in 70% ethanol were pretreated with 0.1% trypsin at room temperature for 20 min and incubated overnight at 4°C with rat antibody against mouse CD31 (PECAM-1; PharMingen, San Diego, CA). Sections then were incubated with biotinylated rabbit anti-rat IgG antibody (DAKO, Glostrup, Denmark) followed by streptavidin-biotin-peroxidase complex, as described previously.[20] The microvessel density was evaluated by counting CD31/PECAM-1-positive vessels using a light microscope at 200-fold magnification in ≥10 randomly selected fields at the periphery of each section.

Proliferating cells were detected using antibody against proliferating cell nuclear antigen (PCNA). Tissue sections were boiled with 10 m*M* citrate buffer (pH 6.0) for 20 min and incubated with mouse anti-PCNA antibody (PC-10; NeoMarkers, Fremont, CA) for 30 min at room temperature. Then, sections were incubated using the DAKO mouse Envision Plus visualization system for 30 min.

Apoptotic cells were detected by a terminal deoxynucleotidyl transferase (Tdt)-mediated nick-end labeling (TUNEL) assay. The *in situ* Apoptosis Detection Kit (TaKaRa Biomedical, Otsu) was used according to the manufacturer's instructions.

Numbers of TUNEL-positive apoptotic cells and PCNA-positive proliferating cells were determined by counting at least 1000 nuclei in different sections. Apoptotic index (%) and proliferation index (%) were defined as percentages of cells evaluated as positive.

To analyze expression of murine HGF, tissue sections were pretreated with 0.1% trypsin at room temperature for 30 min and incubated overnight at 4°C with rabbit anti-rat HGFα chain antibody (Immuno Biological Laboratories, Gunma). Sections then were incubated with the DAKO rabbit Envision Plus visualization system for 30 min.

Measurement of murine HGF in culture media. Primary mouse fibroblasts were obtained from dermal tissues in culture where fibroblasts initially proliferated outward from the tissues. These cells were cultured in RPMI 1640 supplemented with 10% FBS. To prepare conditioned media (CM) from CT26-NK4 and CT26-NEO, confluent cells were washed three times and incubated in RPMI 1640 supplemented with 1% FBS in the presence of 10 ng/ml HGF for 3 days. Mouse fibroblasts were seeded on 24-well plates at a density of 1×10^5 cells/well and cultured for 24 h. After replacing the media with fresh RPMI 1640 supplemented with 1% FBS, 50% CM (v/v) from the transfectants were added and the cells were cultured for 24 h. The concentration of murine HGF in the culture media was determined by ELISA.

RNA isolation and semiquantitative RT-PCR. Expression of IL-1β, bFGF, PDGF, and TGF-α mRNA in each transfectant was

examined by semiquantitative RT-PCR. Subconfluent CT26-NK4 or CT26-NEO cells were cultured with RPMI 1640 supplemented with 2% FBS in the presence or absence of 10 ng/ml HGF for 10 h. Total RNA was isolated from these transfectants using an acid guanidinium thiocyanate-phenol (AGTP) solution (Isogen; Nippon Gene, Tokyo) and quantified spectrophotometrically at 260 nm. Two micrograms of total RNA was subjected to reverse transcription using SuperScript II (Gibco BRL, Grand Island, NY). The cDNA was amplified with the following primers: for IL-1β, sense, 5′-ACCTTCTTTTCCTTCATC-3′ and antisense, 5′-GCTTTTCCATCTTCTTCT-3′; for bFGF, sense, 5′-ACACGTCAAACTACAACTCCA-3′ and antisense, 5′-TCAGCTCTTAGCAGACATTGG-3′; for PDGF, sense, 5′-CGAGGAAGCCGAGATAC-3′ and antisense, 5′-CGCTGCTGGTGTTACAA-3′; for TGF-α, sense, 5′-TTGCTAACCCACACCGAA-3′ and antisense, 5′-ACGCCTGACATTGACATA-3′; and for β-actin as an internal control, sense, 5′-CCAACCGTGAAAAGATG-3′ and antisense, 5′-CCAATAGTGATGACCTG-3′. The number of PCR cycles was determined in a preliminary run to ensure that the target genes were amplified logarithmically. PCR products (10 μl) were resolved on a 1.5% agarose gel and stained with 0.5 μg/ml ethidium bromide. For semiquantitative analyses of IL-1β, bFGF, PDGF, and TGF-α mRNA in each cell sample, images of gels were obtained using an ultraviolet image analyzer. Densities of product bands were quantified using the NIH Image program (version 1.63; Division of Computer Research and Technology, National Institutes of Health, Bethesda, MD). Expression of each target gene was standardized with control gene expression and represented as a ratio. These RT-PCR assays were performed independently three times.

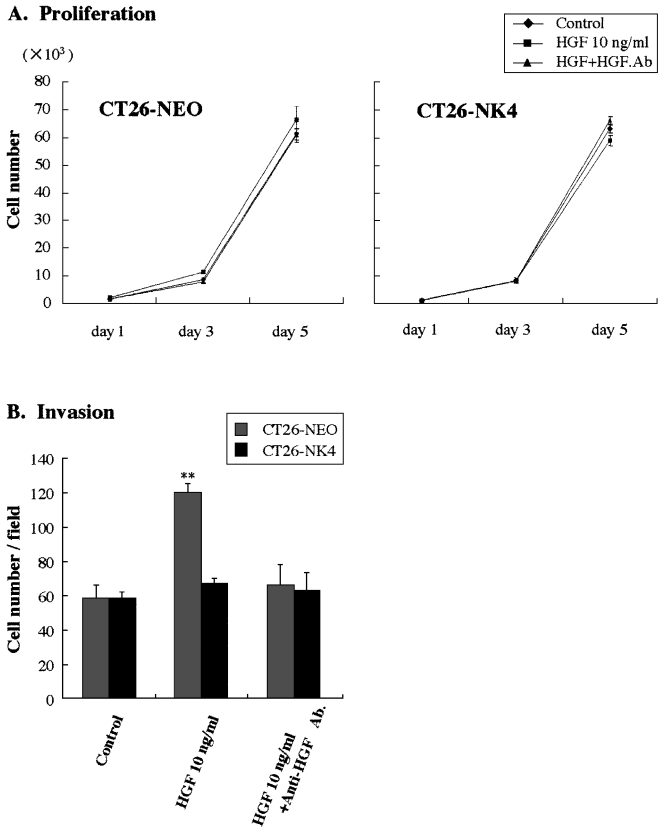


Fig. 1. *In vitro* effects of HGF (10 ng/ml) on malignant properties of CT26-NK4 and CT26-NEO. A: HGF showed slight and insignificant stimulation of cell proliferation in CT26-NEO, but had no such effect in CT26-NK4. B: HGF significantly stimulated invasion by CT26-NEO, but not by CT26-NK4. HGF stimulation of proliferation (A) and invasion (B) by CT26-NEO was completely inhibited by addition of 5 μg/ml anti-HGF antibody. Each error bar denotes the standard deviation (** $P<0.01$).

Statistical analysis. Statistical analyses were performed with unpaired two-tailed Student's *t* tests unless otherwise specified. Differences were considered to be statistically significant at $P<0.05$.

Results

Production of NK4 and murine HGF by transfectants. We measured NK4 and murine HGF concentrations by ELISA in culture media from transfectants and parental CT26 cells. NK4 production by CT26-NK4 was 5.35 ng/10^6 cells/24 h, while CT26-NEO and parental CT26 cells produced no detectable NK4. Murine HGF was not detected in media from transfectants or parental CT26 cells.

Inhibition of HGF-induced cell proliferation, invasion, and colony scattering by NK4. We next analyzed the effect of HGF on *in vitro* biological activities of the transfectants. As regards *in vitro* cell proliferation, HGF caused only a slight and insignificant stimulation of proliferation in CT26-NEO (day 5; 66.0±4.8 (×10^3 cells) with HGF vs. 61.3±2.1 without HGF, $P>0.05$). HGF had no effect on proliferation of CT26-NK4 (Fig. 1A). In the invasion assay, cell invasion was doubled by 10 ng/ml HGF (120.0±5.0 cells with HGF vs. 58.6±7.5 without HGF, $P<0.01$) in CT26-NEO, while this effect was not observed in CT26-NK4 (Fig. 1B). The stimulatory effect of HGF on cell invasion observed in CT26-NEO was completely inhibited by addition of anti-HGF antibody in 500-fold excess over HGF (Fig. 1B). Moreover, HGF is known to be a potent stimulator of tumor cell colony dissociation, as well as epithelial cells, which results in cell scattering.[34, 35] Then, we tested the effect of HGF on scattering of transfectants in monolayer culture. The cell scattering of CT26-NEO was evidently stimulated by the addition of 10 ng/ml HGF, while that of CT26-NK4 was unaffected (Fig. 2). These findings indicate that NK4 expression inhibits the biological activities of CT26 cells stimulated by HGF via an HGF/c-Met-dependent pathway.

Suppression of tumor growth by NK4. After noting inhibition of HGF biological activities by NK4 expression *in vitro*, we examined whether NK4 expression could inhibit tumor growth and prolong survival *in vivo*. In preliminary animal experiments, we analyzed NK4 in transplanted tumors, blood, liver, spleen, and kidney. Considerable NK4 was detected in CT26-NK4 tumors, amounting to a mean value of 128.14 ng/g of tissue on day 7 and 34.46 ng/g of tissue on day 14; little NK4 was detectable in other organs. To investigate the effect of NK4 expression on tumor growth *in vivo*, CT26-NK4 or CT26-NEO was inoculated s.c. into syngeneic mice. As shown in Fig. 3, while CT26-NEO tumors maintained growth, the growth of CT26-NK4 tumors was potently suppressed (day 42; 73.6% inhibition compared with CT26-NEO tumors, $P<0.01$). Furthermore, 6 of 10 mice bearing CT26-NK4 tumors showed complete tumor regression, and survival of mice bearing CT26-NK4 tumors was significantly prolonged ($P<0.01$; Fig. 4). In contrast, all mice bearing CT26-NEO tumors died within 72 days.

Histopathological analyses of implanted tumors. In the immunohistochemical analysis of PCNA, the proliferation index in CT26-NK4 tumors was only 89.3% of that in control tumors (70.8±2.8% in CT26-NK4 vs. 79.3±2.3% in CT26-NEO, $P<0.05$; Fig. 5A), even though no significant difference had been seen in cell proliferation *in vitro* between CT26-NK4 and CT26-NEO. The apoptotic index in CT26-NK4 tumors was 16 times higher than that in control tumors (7.77±0.78% vs. 0.49±0.09%, $P<0.001$; Fig. 5B).

For analysis of tumor vascularity, tumor microvasculature was visualized using anti-CD31/PECAM-1 antibody. The num-

ber of CD31/PECAM-1-positive microvessels at the periphery of CT26-NK4 tumors was only 60% of the number in control tumors (5.9±0.57 vessels/field in CT26-NK4 vs. 9.7±0.52 in CT26-NEO, $P<0.001$; Fig. 5C).

HGF is a critical molecule in tumor-stromal interactions, conferring invasive potential upon tumor cells. To assess whether NK4 affects these tumor-stromal interactions, HGF expression was evaluated by staining with anti-rat HGFα chain antibody. While strong in CT26-NEO tumors, HGF expression was considerably reduced in CT26-NK4 tumors (Fig. 6A). The distribution of HGF expression coincided with that of cytokeratin 7/17 (C46), a marker for stromal cells (not shown).

***In vitro* inhibition of mouse fibroblast-derived HGF production by NK4.** Our histological findings for murine HGF suggest that NK4 expression at a tumor site may inhibit stromal HGF production. We then asked whether HGF production from fibroblasts would be affected by NK4 or other factors secreted from CT26 cells *in vitro*. Normal mouse fibroblasts were cultured in the presence of 50% CM derived from the transfectants, and murine HGF concentration in the culture medium was measured. HGF production from fibroblasts was evidently stimulated by CM from CT26-NEO, while it was not stimulated by that from CT26-NK4 (Fig. 6B). These results indicate that CT26 cells secrete factors that stimulate HGF production from stromal fibroblasts, and also suggest that NK4 may act on fibroblasts in a paracrine manner to reduce their HGF production or may act on CT26 cells in an autocrine manner to modulate their production of HGF inducers, leading to a reduction of HGF production from fibroblasts.

Inhibition of mRNA expression of tumor-derived HGF inducers by NK4. As reported previously, IL-1α, IL-1β, bFGF, TGF-α, PDGF, and PGE2 stimulate HGF production from stromal

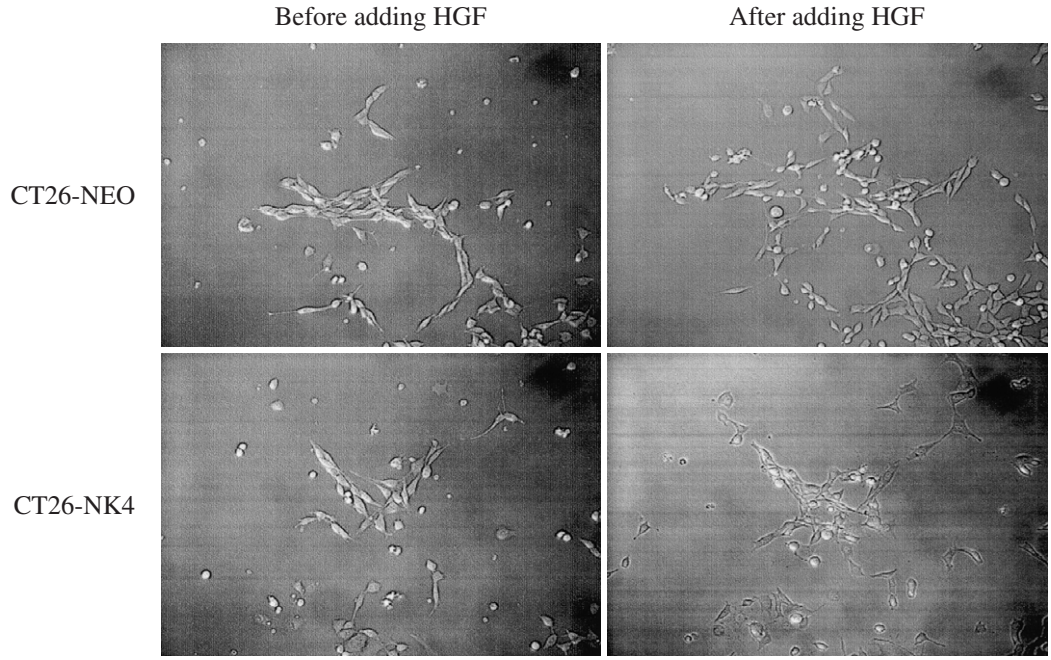


Fig. 2. *In vitro* effect of HGF on colony scattering of transfectants. The colony of each transfectant was microscopically observed 15 h after adding HGF. The cell scattering induced by HGF in CT26-NEO was not observed in CT26-NK4.

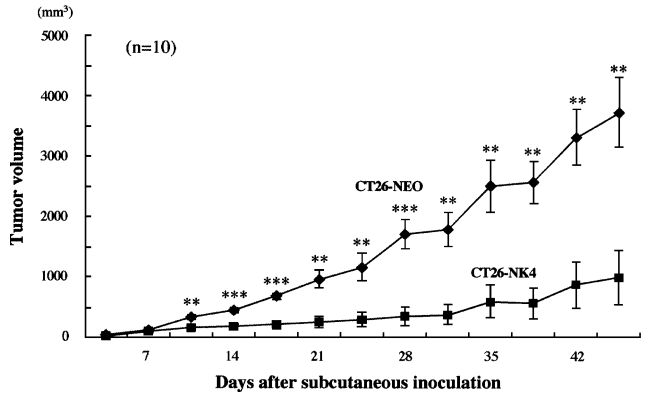


Fig. 3. The effect of NK4 expression on CT26 tumor growth *in vivo*. CT26-NK4 or CT26-NEO (5×10^5 cells) were inoculated s.c. into syngeneic BALB/c mice in the right lower flank, and tumor volume was measured ($n=10$ for each group). Growth of CT26-NK4 tumors was potently suppressed compared with CT26-NEO tumors. Each error bar denotes the standard deviation (** $P<0.01$, *** $P<0.001$).

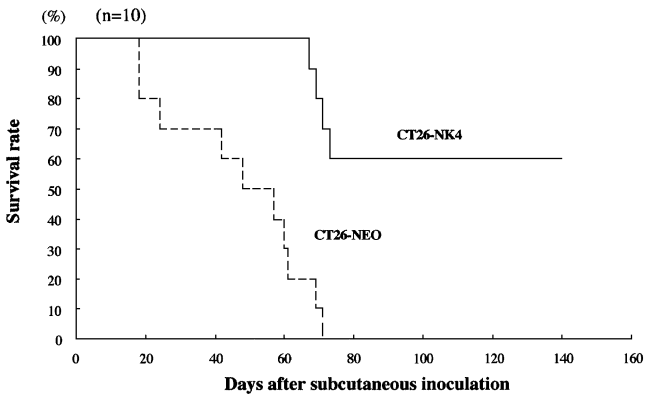


Fig. 4. Survival analysis of the mice bearing CT26-NK4 or CT26-NEO tumors was carried out by the Kaplan-Meier method with comparisons by the log-rank test. The survival of mice bearing CT26-NK4 tumors was significantly prolonged ($P<0.0001$); indeed, 6 of 10 mice showed complete tumor regression.

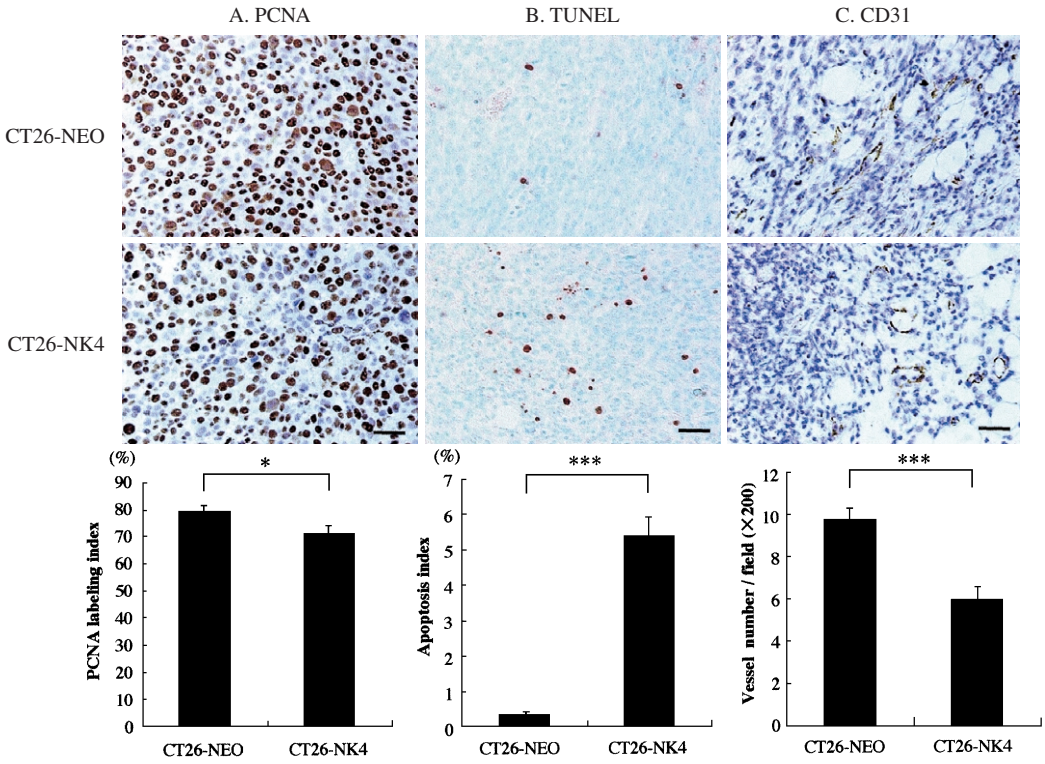


Fig. 5. Immunohistochemical analyses of implanted CT26-NK4 and CT26-NEO tumors. Anti-PCNA antibody, TUNEL assay, and anti-CD31 antibody were used to determine proliferating cells (A), apoptotic cells (B), and angiogenesis (C), respectively. Proliferating cells were decreased and apoptotic cells were markedly increased in CT26-NK4 tumors. The number of microvessels was significantly reduced in CT26-NK4 tumors compared with control tumors. Each error bar denotes the standard deviation (* $P<0.05$, *** $P<0.001$). Scale bar, 100 μm.

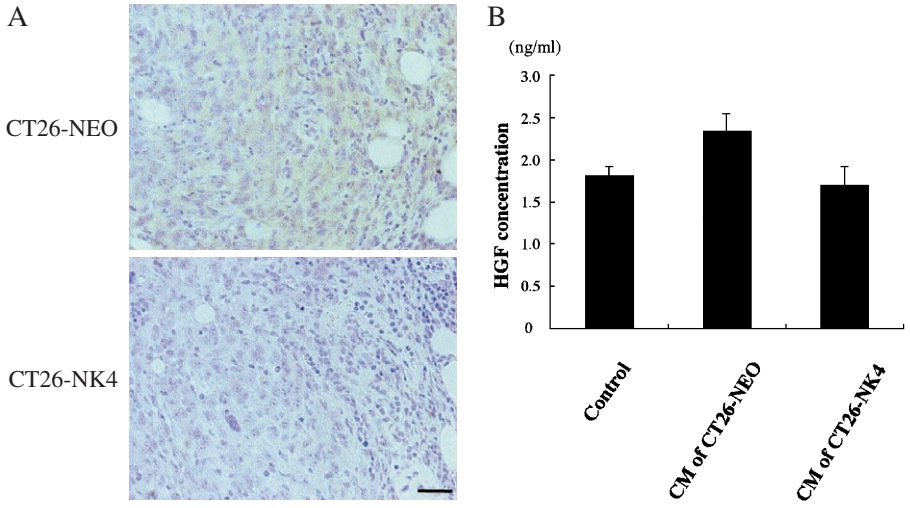
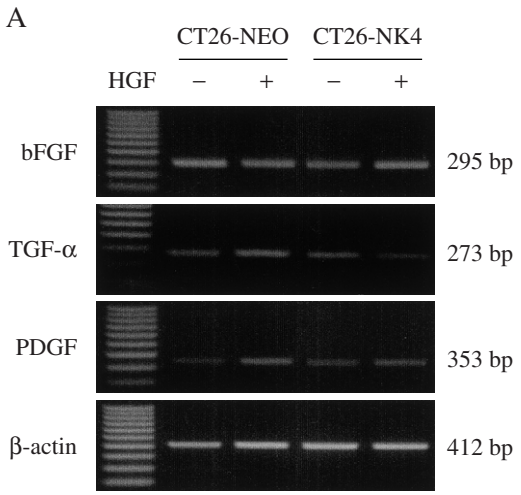


Fig. 6. A: Immunohistochemical analysis of implanted CT26-NK4 and CT26-NEO tumors for murine HGF expression. HGF was expressed strongly in CT26-NEO while expression was reduced in CT26-NK4 tumors. Scale bar, 100 μm. B: Measurement of *in vitro* mouse fibroblast-derived HGF production. Normal mouse fibroblasts were cultured in the presence of 50% conditioned media derived from the transfectants and the HGF concentration in the culture medium was measured. The addition of CM from mock transfectant stimulated murine HGF production and the stimulatory effect was blocked by NK4 expression. The data represent mean±SD of triplicate assays.

fibroblasts.[1–5] To test the hypothesis that the decreased stromal HGF expression in CT26-NK4 tumors might be a result of inhibition of HGF inducers by NK4, we examined the expression levels of bFGF, IL-1β, PDGF, and TGF-α mRNA in CT26-NK4 and CT26-NEO cells by RT-PCR in the presence or absence of HGF. Fig. 7 shows the PCR product bands for bFGF, PDGF, TGF-α, and β-actin (A), and also the results of semi-quantitative analysis for each of these HGF inducers (B). CT26-NEO constitutively expressed bFGF, PDGF, and TGF-α mRNA, while CT26-NK4 showed similar levels of expression. IL-1β mRNA was not detectable in either transfectant (data not shown). HGF stimulation enhanced the expression of PDGF and TGF-α mRNAs in CT26-NEO (PDGF, 2.1 times; TGF-α, 1.4 times), suggesting that PDGF and TGF-α act as HGF inducers in CT26 cells. On the other hand, HGF stimulation produced no enhancement of expression of these inducers in CT26-NK4.

Discussion

In this study, we investigated the *in vitro* and *in vivo* biological activities of CT26 cells genetically modified to produce NK4. This NK4 expression inhibited the motility of CT26 cells *in vitro* via an HGF/c-Met-dependent pathway and potently sup-



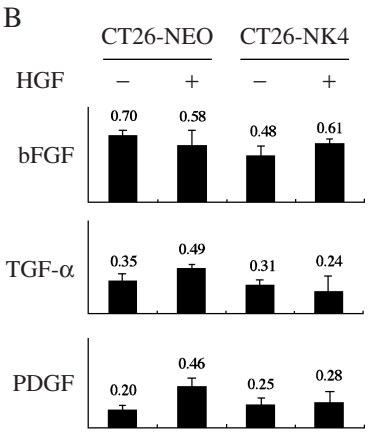


Fig. 7. Expression of mRNAs of HGF inducers (bFGF, PDGF, and TGF-α) in CT26-NK4 and CT26-NEO in the presence or absence of HGF was examined by semiquantitative RT-PCR. CT26-NEO constitutively expressed bFGF, PDGF, and TGF-α mRNA, and CT26-NK4 showed similar constitutive expression. HGF stimulation enhanced expression of PDGF and TGF-α mRNA in CT26-NEO, but not in CT26-NK4. The data represent mean±SD of triplicate assays. Each number is the mean value.

pressed s.c.-implanted tumor growth in syngeneic mice; the growth suppression showed immunohistochemically evident association with angiogenesis inhibition.

NK4 exerts antitumor activities via its bifunctional properties as an HGF antagonist and an angiogenesis inhibitor.[30] Recent studies have emphasized angiogenesis inhibition as the predominant mechanism for *in vivo* antitumor activity of NK4. In experimental treatments of orthotopically implanted pancreatic cancer in nude mice involving repeated intraperitoneal administration of recombinant NK4 or anti-HGF antibody, Tomioka *et al*.[36] reported that an HGF/c-Met-dependent pathway is involved in the initial phase of invasion, while HGF/c-Met-independent angiogenesis inhibition is predominantly involved in NK4 effects against the subsequent phases of extensive invasion and metastasis. Saimura *et al*.[37] found that tumor growth inhibition was induced even by small amounts of NK4 that did not alter the *in vitro* invasive potential of the cells; such results would favor angiogenesis inhibition as the main mechanism for *in vivo* antitumor activities of NK4. Our present findings are consistent with the results of these studies.

We also found by immunohistological analyses that HGF expression was evidently reduced in CT26-NK4 tumors compared with control tumors. Furthermore, we demonstrated in an *in vitro* experiment that NK4 production from CT26 cells is closely related to the inhibition of HGF production from fibroblasts. Previous reports have demonstrated close association of HGF/c-Met signaling responsible for tumor-stromal interactions with malignant phenotypes of tumor cells in experimental models. Giordano *et al*.[38] found that after transfection with human c-Met cDNA, mouse NIH 3T3 fibroblasts expressed a functional c-Met receptor and acquired *in vitro* invasiveness and *in vivo* tumorigenecity. Lamszus *et al*.[39] reported that human breast carcinoma cells genetically modified to produce HGF exhibited more extensive angiogenesis and enhanced tumor growth in nude mice than was seen with parental cells. Therefore, our *in vivo* and *in vitro* results suggest that HGF derived from stromal cells is an essential factor for malignant progression of CT26 tumor, and that inhibition of stromal HGF production is involved in suppression of tumor growth induced by NK4.

Tumor-stromal interactions involve not only stromal-derived HGF, but also tumor-derived HGF inducers.[1–5] Therefore, to clarify the mechanism of stromal HGF inhibition in CT26-NK4 tumors *in vivo*, we investigated *in vitro* expression of HGF inducers by the transfectants. RT-PCR results showed that bFGF, PDGF, and TGF-α were expressed constitutively by CT26 cells; among these HGF inducers, expression of PDGF and TGF-α was stimulated by HGF. NK4 expression as a consequence of gene transfer did not affect constitutive expression of these HGF inducers, but it inhibited the enhancement of PDGF and TGF-α expression in response to HGF. This suggests that HGF promotes not only motility of tumor cells, but also secretion of HGF inducers by tumor cells, representing HGF amplification *in vivo* by mutual stimulation between stromal-derived HGF and tumor-derived HGF inducers, and NK4 reduces the malignant potential not only by antagonizing HGF, but also by subsequently inhibiting HGF amplification.

The present data suggest that angiogenesis inhibition not only by direct action of NK4, but also by its indirect action through endogenous HGF suppression, as well as its antagonism of HGF may play a crucial role in the antitumor effects induced by continuous and high levels of NK4 produced from tumor cells by gene transfer. However, it seems difficult to explain the high frequency of complete regression in CT26-NK4 tumors solely in terms of angiogenesis inhibition and HGF antagonism by NK4. Significant inhibition of cell proliferation *in vivo* observed in CT26-NK4 tumors may also contribute to the antitumor effects, although the proliferation rates of CT26-NK4 and CT26-NEO *in vitro* were almost equal. Importantly, previous reports demonstrated no significant difference in proliferation *in vitro* or even *in vivo* between NK4-treated and control tumor cells, even in the presence of HGF. This suggests that other factors different from angiogenesis inhibition and HGF antagonism may be involved in the present antitumor effects induced by NK4. Further studies will be required to elucidate in detail the antitumor mechanisms of NK4.

In using angiogenesis inhibitors such as NK4 for cancer treatment, continuous exposure of cancer cells to the agent is required for prevention of invasion and metastasis. This may be achieved by using continuous infusion pumps,[29, 30] by altering the agents to prolong high plasma concentrations, or by gene transfer.[40, 41] We have demonstrated that NK4 gene transfer reduced tumor growth and angiogenesis in a cancer cell line implanted into mice, and that the NK4 expression influenced tumor-stromal interactions to reduce stromal-derived HGF production. Our findings emphasize the promise of NK4 gene transfer for anticancer therapy.

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1. Nakamura T, Matsumoto K, Kiritoshi A, Tano Y, 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* 1997; **57**: 3305–13.
2. Matsumoto K, Date K, Shimura H, Nakamura T. Acquisition of invasive phenotype in gallbladder cancer cells via mutual interaction of stromal fibroblasts and cancer cells mediated by hepatocyte growth factor. *Jpn J Cancer Res* 1996; **87**: 702–10.
3. Matsumoto K, Okazaki H, Nakamura T. Up-regulation of hepatocyte growth factor gene expression by interleukin 1 in human skin fibroblasts. *Biochem Biophys Res Commun* 1992; **188**: 235–43.
4. Gohda E, Matsunaga T, Kataoka H, Takebe T, Yamamoto I. Induction of hepatocyte growth factor in human skin fibroblasts by epidermal growth factor, platelet-derived growth factor, and fibroblast growth factor. *Cytokine* 1994; **6**: 633–40.
5. Seslar SP, Nakamura T, Byers SW. Regulation of fibroblast hepatocyte growth factor/scatter factor expression by human breast carcinoma cell lines and peptide growth factors. *Cancer Res* 1993; **53**: 1233–8.
6. Nakamura T, Nishizawa T, Hagiya M, Seki T, Shimoniski M, Sugimata A, Tashiro K, Shimizu S. Molecular cloning and expression of human hepatocyte growth factor. *Nature* 1989; **342**: 440–3.
7. Nakamura T, Nawa K, Ichihara A. Partial purification and characterization of hepatocyte growth factor from serum of hepatectomized rats. *Biochem Biophys Res Commun* 1984; **122**: 1450–9.
8. Hiscox SE, Hallett MB, Puntis MC, Nakamura T, Jiang WG. Expression of the HGF/SF receptor, c-met, and its ligand in human colorectal cancers. *Cancer Invest* 1997; **15**: 513–21.
9. Kaji M, Yonemura Y, Harada S, Liu X, Terada I, Yamamoto H. Participation of c-met in the progression of human gastric cancers: anti-c-met oligonucleotides inhibit proliferation or invasiveness of gastric cancer cells. *Cancer Gene Ther* 1996; **3**: 393–404.
10. Iwazawa T, Shiozaki H, Doki Y, Inoue M, Tamura S, Matsui S, Monden T, Matsumoto K, Nakamura T, Monden M. Primary human fibroblasts induce diverse tumor invasiveness: involvement of hepatocyte growth factor as an important paracrine factor. *Jpn J Cancer Res* 1996; **87**:1134–42.
11. Beviglia L, Matsumoto K, Lin CS, Ziober BL, Kramer RH. Expression of the c-Met/HGF receptor in human breast carcinoma: correlation with tumor progression. *Int J Cancer* 1997; **74**: 301–9.
12. Boix L, Rosa JI, Ventura F, Castells A, Bruix J, Rodes J, Bartrons R. c-met messenger RNA overexpression in human hepatocellular carcinoma. *Hepatology* 1994; **19**: 88–91.
13. Hu Z, Evarts RP, Fujio K, Omori N, Omori M, Marsden ER, Thorgeirsson SS. Expression of transforming growth factor alpha/epidermal growth factor receptor, hepatocyte growth factor/c-met and acidic fibroblast growth factor/fibroblast growth factor receptors during hepatocarcinogenesis. *Carcinogenesis* 1996; **17**: 931–8.
14. Harvey P, Warn A, Newman P, Perry LJ, Ball RY, Warn RM. Immunoreactivity for hepatocyte growth factor/scatter factor and its receptor, met, in human lung carcinomas and malignant mesotheliomas. *J Pathol* 1996; **180**: 389–94.
15. Takanami I, Tanana F, Hashizume T, Kikuchi K, Yamamoto Y, Yamamoto T, Kodaira S. Hepatocyte growth factor and c-Met/hepatocyte growth factor receptor in pulmonary adenocarcinomas: an evaluation of their expression as prognostic markers. *Oncology* 1996; **53**: 392–7.
16. Bussolino F, DiRenzo MF, Ziche M, Bocchietto E, Olivero M, Naldini L, Gaudino G, Tamagnone L, Coffer A, Comoglio PM. Hepatocyte growth factor is a potent angiogenic factor which stimulates endothelial cell motility and growth. *J Cell Biol* 1992; **119**: 629–41.
17. Grant DS, Kleinman HK, Goldberg ID, Bhargava MM, Nickoloff BJ, Kinsella JL, Polverini P, Rosen EM. Scatter factor induces blood-vessel formation *in vivo*. *Proc Natl Acad Sci USA* 1993; **90**: 1937–41.
18. Folkman J. Seminars in Medicine of the Beth Israel Hospital, Boston. Clinical applications of research on angiogenesis. *N Engl J Med* 1995; **333**: 1757–63.
19. Hanahan D, Folkman J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* 1996; **86**: 353–64.
20. Weidner N, Semple JP, Welch WR, Folkman J. Tumor angiogenesis and metastasis-correlation in invasive breast carcinoma. *N Engl J Med* 1991; **324**: 1–8.
21. Macchiarini P, Fontanini G, Hardin MJ, Squartini F, Angeletti CA. Relation of neovascularisation to metastasis of non-small-cell lung cancer. *Lancet* 1992; **340**: 145–6.
22. Weidner N, Carroll PR, Flax J, Blumenfeld W, Folkman J. Tumor angiogenesis correlates with metastasis in invasive prostate carcinoma. *Am J Pathol* 1993; **143**: 401–9.
23. Smith-MacCune KK, Weidner N. Demonstration and characterization of the angiogenic properties of cervical dysplasia. *Cancer Res* 1994; **54**: 800–4.
24. Kitadai Y, Haruma K, Tokutomi T, Tanaka S, Sumii K, Carvalho M, Kuwabara M, Yoshida K, Hirai T, Kajiyama G, Tahara E. Significance of vessel count and vascular endothelial growth factor in human esophageal carcinomas. *Clin Cancer Res* 1998; **4**: 2195–200.
25. Takahashi Y, Kitadai Y, Bucana CD, Cleary KR, Ellis LM. Expression of vascular endothelial growth factor and its receptor, KDR, correlates with vascularity, metastasis, and proliferation of human colon cancer. *Cancer Res* 1995; **55**: 3964–8.
26. Date K, Matsumoto K, Shimura H, Tanaka M, Nakamura T. HGF/NK4 is a specific antagonist for pleiotrophic actions of hepatocyte growth factor. *FEBS Lett* 1997; **420**: 1–6.
27. Parr C, Hiscox S, Nakamura T, Matsumoto K, Giang WG. NK4, a new HGF/SF variant, is an antagonist to the influence of HGF/SF on the motility and invasion of colon cancer cells. *Int J Cancer* 2000; **85**: 563–70.
28. Maehara N, Matsumoto K, Kuba K, Mizumoto K, Tanaka M, Nakamura T. NK4, a four-kringle antagonist of HGF, inhibits spreading and invasion of human pancreatic cancer cells. *Br J Cancer* 2001; **84**: 864–73.
29. Date K, Matsumoto K, Kuba K, Shimura H, Tanaka M, Nakamura T. Inhibition of tumor growth and invasion by a four-kringle antagonist (HGF/NK4) for hepatocyte growth factor. *Oncogene* 1998; **17**: 3045–54.
30. Kuba K, Matsumoto K, Date K, Shimura H, Tanaka M, Nakamura T. HGF/NK4, a four-kringle antagonist of hepatocyte growth factor, is an angiogenesis inhibitor that suppresses tumor growth and metastasis in mice. *Cancer Res* 2000; **60**: 6737–43.
31. Davies G, Mason MD, Martin TA, Parr C, Watkins G, Lane J, Matsumoto K, Nakamura T, Jiang WG. The HGF/SF antagonist NK4 reverses fibroblast- and HGF-induced prostate tumor growth and angiogenesis *in vivo*. *Int J Cancer* 2003; **106**: 348–54.
32. Seki T, Ihara I, Sugimura A, Shimonishi M, Nishizawa T, Asami O, Hagiya M, Nakamura T, Shimizu S. Isolation and expression of cDNA for different forms of hepatocyte growth factor from human leukocytes. *Biochem Biophys Res Commun* 1990; **172**: 321–7.
33. Corbett TH, Griswold DP, Roberts BJ, Peckham JC, Schabel FJ Jr. Tumor induction relationships in development of transplantable cancers of the colon in mice for chemotherapy assays, with note on carcinogen structure. *Cancer Res* 1975; **35**: 2434–9.
34. Gherardi E, Gray J, Stoker M, Perryman M, Furlong R. Purification of scatter factor, a fibroblast-derived basic protein that modulates epithelial interaction and movement. *Proc Natl Acad Sci USA* 1989; **86**: 5844–8.
35. Weidner KM, Behrens J, Vanderkerckhove J, Birchmeier W. Scatter factor: molecular characteristics and effect on the invasiveness of epithelial cells. *J Cell Biol* 1990; **111**: 2097–108.
36. Tomioka D, Maehara N, Kuba K, Mizumoto K, Tanaka M, Matsumoto K, Nakamura T. Inhibition of growth, invasion, and metastasis if human pancreatic carcinoma cells by NK4 in an orthotopic mouse model. *Cancer Res* 2001; **61**: 7518–24.
37. Saimura M, Nagai E, Mizumoto K, Maehara N, Minamishima YA, Katano M, Matsumoto K, Nakamura T, Tanaka M. Tumor suppression through angiogenesis inhibition by SUIT-2 pancreatic cancer cells genetically engineered to secrete NK4. *Clin Cancer Res* 2002; **10**: 3243–9.
38. Giordano S, Zhen Z, Medico E, Gaudino G, Galimi F, Comoglio PM. Transfer of motogenic and invasive response to scatter factor/hepatocyte growth factor by transfection of human MET protooncogene. *Proc Natl Acad Sci USA* 1993; **90**: 649–53.
39. Lamszus K, Jin L, Fuchs A, Shi E, Chowdhury S, Yao Y, Polverini PJ, Laterra J, Goldberg ID, Rosen EM. Scatter factor stimulates tumor growth and tumor angiogenesis in human breast cancers in the mammary fat pads of nude mice. *Lab Invest* 1997; **76**: 339–53.
40. Saimura M, Nagai E, Mizumoto K, Maehara N, Okino H, Katano M, Matsumoto K, Nakamura T, Narumi K, Nukiwa T, Tanaka M. Intraperitoneal injection of adenovirus-mediated NK4 gene suppresses peritoneal dissemination of pancreatic cancer cell line AsPC-1 in nude mice. *Cancer Gene Ther* 2002; **9**: 799–806.
41. Maemondo M, Narumi K, Saijo Y, Usui K, Tahara M, Tazawa R, Hagiwara K, Matsumoto K, Nakamura T, Nukiwa T. Targeting angiogenesis and HGF function using an adenoviral vector expressing the HGF antagonist NK4 for cancer therapy. *Mol Ther* 2002; **5**: 177–85.