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Endocrine Delivery System of NK4, an HGF-Antagonist and Anti-Angiogenic Regulator, for Inhibitions of Tumor Growth, Invasion and Metastasis

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1. Introduction

Estimates of the worldwide incidence and mortality from 27 cancers in 2008 have been prepared for 182 countries by the International Agency for Research on Cancer (Ferlay *et al.*, 2010). Overall, an estimated 12.7 million new cancer cases and 7.6 million cancer deaths occur in 2008, with 56% of new cancer cases and 63% of the cancer deaths occurring in the less developed regions of the world. The most commonly diagnosed cancers worldwide are lung (1.61 million, 12.7% of the total), breast (1.38 million, 10.9%) and colorectal cancers (1.23 million, 9.7%). Cancer is neither rare anywhere in the world, nor mainly confined to high-resource countries. Many cancer subjects die from cancer as a result of organ failure due to “metastasis” (Geiger & Peeper, 2009), thus indicating that medical control of tumor metastasis leads to a marked improvement in cancer prognosis.

The acquisition of the metastatic phenotype is not simply the result of oncogene mutations, but instead is achieved through an interstitial stepwise selection process (Mueller & Fusenig, 2004). The dissociation and migration of cancer cells, together with a breakdown of basement membranes between the parenchyme and stroma, are a prerequisite for tumor invasion. The next sequential events involved in cancer metastasis include the following: (i) penetration of cancer cells to adjacent vessels (*i.e.*, intravasation); (ii) suppressed anoikis (*i.e.*, suspension-induced apoptosis) of cancer cells in blood flow; and (iii) an extravascular migration and re-growth of metastatic cells in the secondary organ. For an establishment of anti-metastasis therapy, it is important to elucidate the basic mechanism(s) whereby tumor metastasis is achieved through a molecular event(s).

Hepatocyte growth factor (HGF) was discovered and cloned as a potent mitogen of rat hepatocytes in a primary culture system (Nakamura *et al.*, 1984, 1989; Nakamura, 1991). Beyond its name, HGF is now recognized as an essential organotrophic regulator in almost all tissues (Nakamura, 1991; Rubin *et al.*, 1993; Zarnegar & Michalopoulos, 1995; Birchmeier & Gherardi, 1998; Nakamura & Mizuno, 2010). Actually, HGF induces mitogenic, motogenic

and morphogenic activities in various types of cells via its receptor, MET (Bottaro *et al.*, 1991; Higuchi *et al.*, 1992). HGF is required for organogenesis in an embryonic stage and for tissue repair in adulthood during various diseases (Nakamura, 1991; Birchmeier & Gherardi, 1998; Nakamura & Mizuno, 2010). Several lines of *in vitro* studies indicate that HGF stimulates scattering and migration of cancer cells (Matsumoto *et al.*, 1994, 1996a; Nakamura *et al.*, 1997). In malignant tumors, HGF is expressed by stromal cells, such as fibroblasts, while MET is over-expressed by cancer cells, thus suggesting in the mid-1990s that a paracrine signal from HGF-producing stroma cells to carcinomas may cause malignant behaviors, such as invasion and metastasis (Matsumoto *et al.*, 1996b).

NK4 is an intra-molecular fragment of HGF, which is generated by a chemical cleavage of mature form HGF (Date *et al.*, 1997; Nakamura *et al.*, 2010). NK4 includes an N-terminal hairpin domain and 4-kringle domains (K1-K4) of HGF α -chain, which binds to MET. Thus, NK4 antagonizes HGF activities as a competitive inhibitor. Using NK4 as an HGF-antagonist in rodents with malignant tumors, we have accumulated evidence showing that endogenous HGF-MET cascade is a key conductor for tumor metastasis, while inhibition of MET signals leads to the arrests of tumor growth. Unexpectedly, NK4 prohibits tumor angiogenesis through a MET-independent mechanism. This review focuses on the roles of HGF in cancer biology and pathology. We also emphasize the effectiveness of NK4 in experimental cancer models where NK4 is supplemented via a “hydrodynamics-based” gene therapy.

2. Effects of HGF on intra-tumor cells during cancer progression

In the mid-1980s, MET was identified as a mutated oncogene from carcinogen-induced osteosarcoma cells (MNNG-HOS) that transform NIH3T3 fibroblasts (Cooper *et al.*, 1984). MET-encoding protein has a tyrosine kinase activity (Dean *et al.*, 1985), suggesting that MET may be an orphan receptor of growth factors. In the early 1990s, MET-coding product was demonstrated to be a high-affinity receptor for HGF (Bottaro *et al.*, 1991; Higuchi *et al.*, 1992). Scatter factor (SF) stimulates tumor cell movement, as its name indicates, and is shown molecularly identical to HGF (Konishi *et al.*, 1991; Weidner *et al.*, 1991). HGF has several activities required for tumor cell invasion and metastasis, as described below. In this section, we summarize the direct effects of HGF on intra-tumor cells, including carcinoma, and on vascular and lymphatic cells prior to discussion of the contribution of HGF-MET cascades during tumor malignancy.

2.1 Scattering and migration of tumor cells

Initial events for the metastatic spread of tumors involve loss of cell-cell contact within the primary tumor mass. The integrity and morphology of epithelial tumor cell colonies are maintained by cell-cell contact mediated by cadherins and its associated intracellular catenin molecules. Cancer cells must lose their tight cell-to-cell contact by down-regulation of cadherin-cadherin complex during invasion into adjacent tissues. HGF induces scattering (*i.e.*, dispersion of cluster cells into single cells) via an endocytosis of E-cadherin from cell surface to cytoplasm (Watabe *et al.*, 1993; Miura *et al.*, 2001). During cell migration, HGF activates the Ras-Rab5 pathway for endocytosis of cadherins (Kimura *et al.*, 2006), which triggers nuclear localization of β -catenin, a transcription factor of genes responsible for cell motility (Hiscox & Jiang, 1999). Stimulation of an Rho small G protein cascade and activation of cdc42, rac and PAK by HGF leads to the disassembly of stress fiber or focal adhesions, while lamellipodia

formation and cell spreading are enhanced by HGF (Royal *et al.*, 2000). These changes confer a down-stream mechanism of MET-mediated cancer invasion.

2.2 Breakdown of basement membranes

During cancer invasion, tumor cells must move across a basement membrane between epithelium and lamina propria (*i.e.*, sub-epithelium). HGF stimulates motility in a biphasic process: cells spread rapidly and form focal adhesions, and then they disassemble these condensations, followed by increased cell locomotion. In the early phase (*i.e.*, within a few minutes post-stimulation), HGF induces phosphorylation of focal adhesion kinase (FAK) together with a tight bridge between the extra-cellular matrix (ECM) and integrins of cancer cells (Matsumoto *et al.*, 1994; Parr *et al.*, 2001). In the later phase, HGF-stimulated cancer cells invade into matrix-based gels *in vitro*, or across basement membrane ECM *in vivo* (Nakamura *et al.*, 1997). In this process, HGF up-regulates several types of matrix metalloproteinase (MMP), such as MMP-1, -2, and -9, through activation of Ets, a transcriptional factor of MMPs (Li *et al.*, 1998; Nagakawa *et al.*, 2000; Jiang *et al.*, 2001). Considering that MMP-inhibitors diminish HGF-mediated migration, the induction of MMP through HGF-Ets cascade is essential for tumor invasion into adjacent normal tissues.

2.3 Endothelial attachment and extravasation of cancer cells

Needless to say, tumor angiogenesis as well as lymphatic vessel formation are important for delivery of cancer cells from the primary tumor to secondary organs. HGF enhances angiogenesis via induction of the proliferation and morphogenesis of endothelial cells (EC) (Bussolino *et al.*, 1992; Nakamura *et al.*, 1996). Actually, HGF supplementation leads to the enhancement of tumor angiogenesis *in vivo* (Lattera *et al.*, 1997). Recent studies delineated the capacity of HGF to induce lymphatic morphogenesis (Kajiya *et al.*, 2005; Saito *et al.*, 2006). Thus, HGF is considered to facilitate cancer metastasis via neo-induction of vascular or lymphatic vessel beds. HGF has a direct effect on EC for enhancing tight adhesion of tumor cells on endothelium via FAK phosphorylation (Kubota *et al.*, 2009a). Furthermore, HGF decreases endothelial occludin, a cell-cell adhesion molecule (Jiang *et al.*, 1999a). Under such a loss of EC-EC integrity, HGF decreases the trans-endothelial resistance of tumor vessels and enhances cancer invasion across an EC barrier (*i.e.*, intravasation in primary tumors and extravasation in metastatic organs) (Fig. 1).

2.4 Prevention of cancer cell anoikis

Anoikis, also known as suspension-induced apoptosis, is a term used to describe programmed cell death (apoptosis) of epithelial cells induced by loss of matrix attachment. In addition to gaining functions of invasion and angiogenesis, cell resistance to anoikis also appears to play an important role in tumor progression and metastasis as tumor cells lose matrix attachment during metastasis. However, it is unknown how cancer cells escape from anoikis-like death during metastasis. It was demonstrated, in a non-adherent culture models, that HGF is a key molecule inhibiting suspension-induced anoikis, and this effect is mediated via a crosstalk that is, in turn, mediated by phosphatidylinositol 3-kinase (PI-3K) and extracellular signal-regulated kinase (ERK)-1/2 (Zeng *et al.*, 2002; Kanayama *et al.*, 2008). A recent report described that tetraspanin CD151-knockdown abolishes preventive effect of HGF on tumor anoikis (Franco *et al.*, 2010). Thus, it is likely that cell surface tetraspanins are important for signaling complexes between MET and integrin- β 4, a known amplifier of HGF-mediated cell survival.

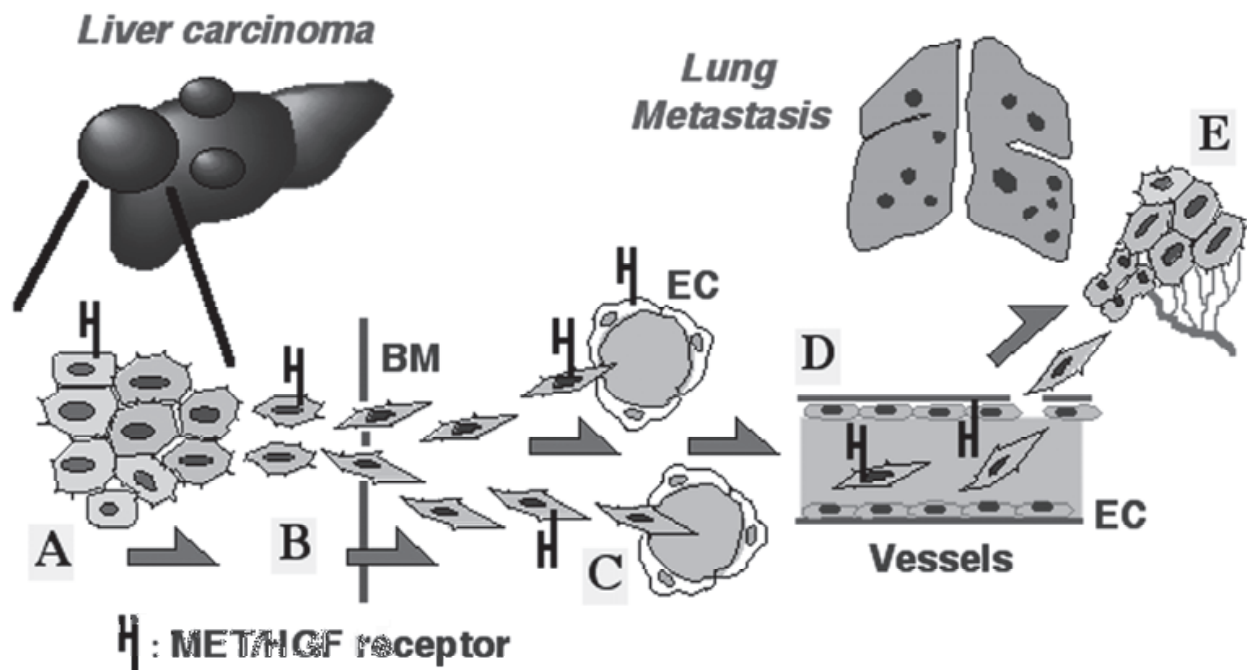


Fig. 1. Various effect of HGF on cancer cells and endothelial cells (EC) during tumor progression. For example, sequential events during the lung metastasis of hepatic carcinoma are summarized as follows: (A) dissociation and scattering of hepatocellular cancer cells through an HGF-induced endocytosis of cadherins; (B) tumor migration into stromal areas across the basement membrane (BM) is mediated via MMP-dependent matrix degradation and Rho-dependent cell movement; (C) invasion of tumor cells into neighboring vessels (*i.e.*, intravasation) where the tight junction between ECs is lost by HGF-MET signaling; (D) inhibition of tumor cell anoikis by MET-AKT cascades during blood flow, and out-flux of tumor cells across vessel walls (*i.e.*, extravasation); and (E) in the lung, HGF supports growth of metastatic nodules via providing vascular beds as an angiogenic factor.

Overall, HGF is shown to take direct action on carcinoma cells: (i) cell spreading via an endocytosis of cadherins; (ii) enhancement of invasion across basement membranes via Rho-dependent and MMP-dependent pathways; and (iii) anti-anoikis activity during blood circulation. Toward tumor vessels, HGF elicits vascular and lymphatic EC proliferation and branching angiogenesis, while intravasation and extravasation are achieved through HGF-induced reduction of EC-EC integrity. These HGF-MET-mediated biological functions seem advantageous for invasion and metastasis of malignant tumors, including carcinoma and sarcoma (Fig. 1).

[Note] Long-term administration of recombinant HGF does not elicit tumor formation in healthy animals, and this result supports a rationale of HGF supplement therapy for treating chronic organ diseases, such as liver cirrhosis, at least in cancer-free patients.

3. Regulation of HGF production by cancer cells

Several lines of histological evidence indicate that HGF is produced in stroma cells, such as fibroblasts, vascular EC and smooth muscle cells in tumor tissues. In contrast, MET is over-expressed mainly by tumor cells, particular near invasive areas, implying a possible paracrine signal from HGF-producing stroma cells to MET-expressing carcinoma cells

(Matsumoto *et al.*, 1996b). Herein, we will discuss the molecular basis whereby stromal HGF production is up-regulated by tumor cells during cancer invasion and metastasis.

3.1 Stroma as a microenvironment to determine behaviors of tumors

The important roles of stroma during tumor progression are demonstrated through several independent studies. Carcinoma-associated fibroblasts, but not normal fibroblasts, stimulate tumor progression of initiated non-tumorigenic epithelial cells both in an *in vivo* tissue recombination and in an *in vitro* co-culture system (Olumi *et al.*, 1999). Transforming growth factor (TGF)- β signaling is critical for down-regulating HGF production (Matsumoto *et al.*, 1992). Of note, an inactivation of TGF- β type II receptor gene in stromal fibroblasts leads to the onset of epithelial growth and invasion (Bhowmick *et al.*, 2004). In this process, activation of paracrine HGF is a key mechanism for stimulation of epithelial proliferation (Bhowmick *et al.*, 2004). Thus, the suppression of HGF production by TGF- β seems to be important for an escape from cancer metastasis (Matsumoto & Nakamura, 2006).

3.2 Regulation of HGF production in stroma by tumor cells

As repeated, a major source of HGF in tumors is stromal cells (including fibroblasts, endothelium, macrophages and neutrophils) (Wislez *et al.*, 2003; Matsumoto & Nakamura, 2006; Grugan *et al.*, 2010). Thus, how stromal HGF is up-regulated during tumor progression should be discussed. There is now ample evidence that numerous types of carcinoma cells secrete soluble factors that induce HGF production in stromal cells (*i.e.*, HGF-inducers). For example, conditioned medium obtained from breast cancer cells enhances HGF production in fibroblasts, along with a raise in prostaglandin-E2 (Matsumoto-Taniura *et al.*, 1999). Of note, suppression of prostaglandin-E2 production by indomethacin leads to down-regulation of stromal HGF production and suppression of tumor migration *in vitro* (Matsumoto-Taniura *et al.*, 1999), indicating that cancer-derived prostaglandins are important for up-regulating HGF in stromal cells (Matsumoto-Taniura *et al.*, 1999; Pai *et al.*, 2003). Other carcinoma-derived HGF-inducers are interleukin-1 β (IL-1 β), basic fibroblast growth factor (b-FGF), platelet-derived growth factor (PDGF), and TGF- α (Hasina *et al.*, 1999; Matsumoto & Nakamura, 2003). These results indicate a crosstalk between carcinoma and stroma, mediated via a paracrine loop of HGF-inducers produced by carcinoma and HGF secreted from stroma cells, such as fibroblasts (Matsumoto *et al.*, 1996a).

3.3 Inflammation-mediated HGF up-regulation mechanism

In addition to stromal fibroblasts, tumor-associated macrophages (TAM) are known to highly produce HGF during non-small lung cancer invasion (Wang *et al.*, 2011). It is reported that TAM isolated from 98 primary lung cancer tissues show the higher production of HGF, along with the concomitant increases in urokinase-type plasmin activator (uPA), cyclooxygenase-2 (Cox2) and MMP-9 (Wang *et al.*, 2011). Anti-MMP-9 antibody largely diminishes TAM-induced invasion, while Cox2 and uPA are critical for HGF production and activation, respectively, suggesting that Cox2-uPA-HGF-MMP cascades in TAM participate in non-small lung cancer invasion. Likewise, HGF production is enhanced by neutrophils infiltrating bronchiolo-alveolar subtype pulmonary adenocarcinoma (Wislez *et al.*, 2003).

Clinical studies demonstrate that serum levels of HGF are elevated in patients with recurrent malignant tumors (Wu *et al.*, 1998; Osada *et al.*, 2008), thus suggesting an

endocrine mechanism of the HGF delivery system. In this regard, it is known that peripheral blood monocytes produce HGF, contributing to the increase in blood HGF levels via an endocrine mechanism (Beppu *et al.*, 2001). Overall, production of HGF by inflammatory cells is involved in carcinoma invasion and metastasis (*i.e.*, local system), while peripheral blood monocytes seem to prevent tumor cell anoikis during metastasis, possibly by a release of HGF into blood (*i.e.*, systemic system).

4. Structure and activity of NK4 as HGF antagonist

HGF is a stromal-derived paracrine factor that has stimulated cancer invasion at least *in vitro* (Matsumoto *et al.*, 1994; Matsumoto *et al.*, 1996a; Nakamura *et al.*, 1997). Clinical studies suggest that the degree of serum HGF and Met expressions in cancer tissues appears to correlate with a given prognosis (Yoshinaga *et al.*, 1993; Osada *et al.*, 2008). Thus, it is hypothesized that *in vivo* inhibition of HGF-MET signaling may be a reasonable strategy to prohibit cancer metastasis. To test this hypothesis, we prepared NK4 as an intra-molecular fragment of HGF via a chemical digestive process (Date *et al.*, 1997; Matsumoto *et al.*, 1998). As expected, NK4 bounded to MET and inhibited HGF-MET coupling as a competitive inhibitor. An additional “unexpected” value was that NK4 inhibited tumor angiogenesis via a MET-independent pathway. This section focuses on the biological value of NK4 as an HGF-antagonist and as an angiogenesis inhibitor.

4.1 Structure and anti-invasive function of NK4

NK4 was initially purified as a fragment from elastase-digested samples of recombinant human HGF (Date *et al.*, 1997). The N-terminal amino acid sequence of NK4 and of the remnant fragment, assumed to be composed of an HGF β -chain, revealed that NK4 is cleaved between the 478th valine and the 479th asparagine. The N-terminal amino acid sequence of NK4 revealed that the N-terminal structure of NK4 is the same as undigested HGF (*i.e.*, 32nd pyroglutamate), indicating that NK4 is composed of the N-terminal 447 amino acids of the α -chain of HGF and contains the N-terminal hairpin domain and four kringle domains (thus designated NK4) (Fig. 2A). The binding domains that are responsible for high-affinity binding to MET are the N-terminal hairpin and the first kringle domains in NK4 (and HGF). MET tyrosine phosphorylation occurs in A549 lung carcinoma within 10 minutes after HGF addition, while NK4 inhibits the HGF-mediated MET activation (Fig. 2B). Actually, NK4 functions as an HGF-antagonist: HGF induces invasion and migration of the gallbladder and bile duct carcinoma cells in ECM-based gels, while NK4 inhibits HGF-induced invasion in a dose-dependent manner (Fig. 2C) (Date *et al.*, 1998). These anti-invasive effects of NK4 are seen in distinct types of cancer cells (Hiscox *et al.*, 2000; Maehara *et al.*, 2001; Parr *et al.*, 2001), strengthening the common role of NK4 during cancer migration.

4.2 Perlecan-dependent anti-angiogenic mechanism by NK4

Vascular EC highly express MET, while HGF stimulates mitogenic and morphogenic activities in EC (Nakamura *et al.*, 1996), thus suggesting that NK4 could inhibit HGF-induced angiogenesis. Actually, NK4 potently inhibited the HGF-mediated proliferation of EC *in vitro* (Jiang *et al.*, 1999b). Strikingly, NK4 also inhibited microvascular EC proliferation and migration, induced by other angiogenic factors, such as b-FGF and vascular endothelial

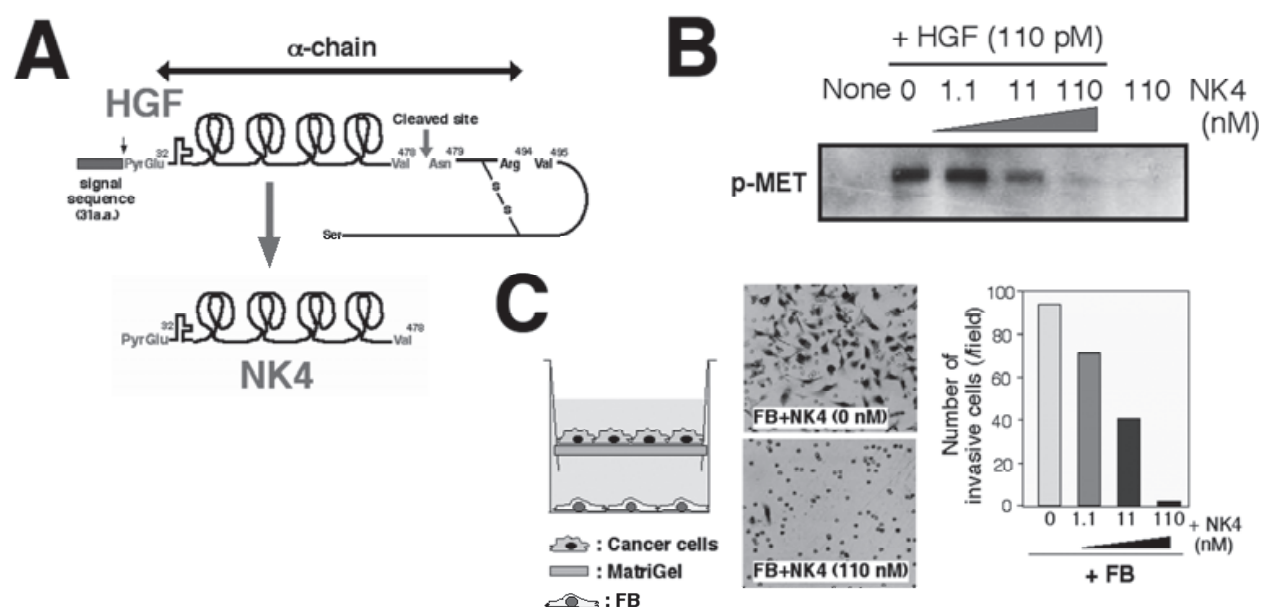


Fig. 2. Preparation of NK4 as an HGF-antagonist and its inhibitory effects on tumor invasion *in vitro*. (A) Preparation and structure of NK4. NK4 is generated via a cleavage of HGF between 478th Val and 479th Asn. (B) Inhibition of HGF-mediated MET tyrosine phosphorylation by NK4 in lung carcinoma cells. (C) Biological activity of NK4. Cancer cell invasion (upper chamber) is induced across a Matrigel layer when fibroblasts (FB) are placed on a lower chamber. In this co-culture system, NK4 inhibits FB-induced tumor cell invasion in a dose-dependent manner.

growth factor (VEGF) (**Fig. 3A**) (Kuba *et al.*, 2000). When a pellet containing b-FGF was implanted under the rabbit cornea, angiogenesis was rapidly induced. In this model, NK4 inhibited b-FGF-induced angiogenesis (**Fig. 3B**). *In vitro* models of EC proliferation, HGF and VEGF phosphorylate MET and KDR/VEGF receptor, respectively, whereas NK4 inhibits HGF-induced MET tyrosine phosphorylation, but not VEGF-induced KDR phosphorylation (Kuba *et al.*, 2000). Nevertheless, NK4 inhibited the VEGF-mediated EC proliferation without modification of VEGF-mediated ERK1/2 (p44/42 mitogen-activated protein kinase) activation. These results suggest the presence of another mechanism whereby NK4 inhibits VEGF- and b-FGF-mediated angiogenesis.

The fibronectin-integrin signal is essential for the spreading and proliferation of EC. Based on this background, we demonstrated that NK4-mediated growth arrest of EC is due to a loss of the fibronectin-integrin signal. Affinity purification with NK4-immobilized beads revealed that NK4 binds to perlecan (Sakai *et al.*, 2009). Consistent with this result, NK4 was co-localized with perlecan in EC. Perlecan is a multi-domain heparan sulfate proteoglycan that interacts with basement membrane components such as fibronectin. Of interest, knockdown of perlecan expression by siRNA diminished the fibronectin assembly and EC spreading, indicating an essential role of fibronectin-perlecan interaction during EC movement. A recent report described that NK4-perlecan interaction suppressed the normal assembly of fibronectin by perlecan (Sakai *et al.*, 2009). As a result, FAK activation became faint in EC after NK4 treatment. Under such a loss of fibronectin-integrin signaling by NK4, EC growth and motility were suppressed, even in the presence of b-FGF or VEGF. This is the reason why NK4 arrests b-FGF- or VEGF-mediated angiogenesis (**Fig. 3C**).

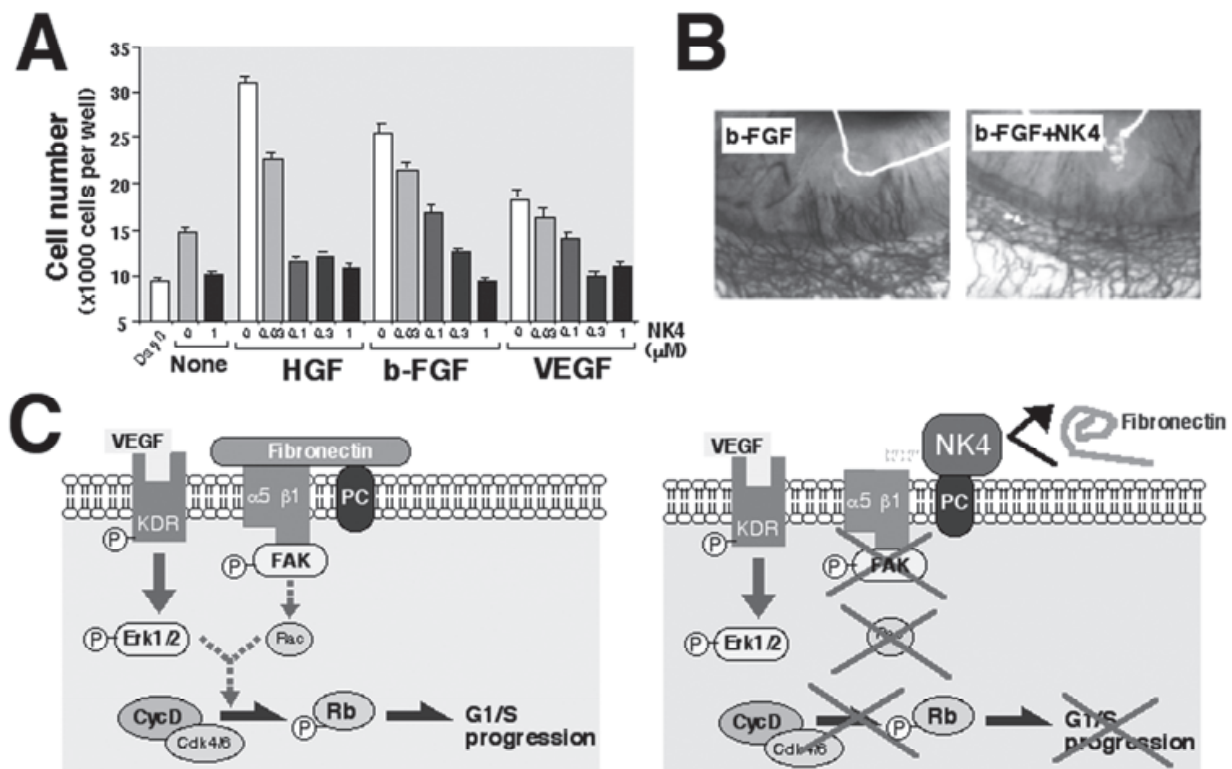


Fig. 3. Anti-angiogenic effects of NK4 via a perlecan-dependent mechanism. (A) NK4 suppresses HGF-, b-FGF-, and VEGF-induced proliferation of EC *in vitro* (Kuba *et al.*, 2000). (B) Inhibition of b-FGF-induced corneal neovascularization by NK4 treatment in rabbits. (C) Involvement of perlecan (PC) in NK4-mediated growth arrest of EC. Left: Cell surface PC is required for the binding of fibronectin and $\alpha 5 \beta 1$ -integrin, leading to FAK phosphorylation and crosstalk of VEGF-VEGF receptor (KDR) signaling. Right: NK4 binds to PC, and then the binding of integrin to fibronectin is impaired. As a result, VEGF fails to elicit G1/S progression of EC in the presence of NK4 (Sakai *et al.*, 2009).

We have accumulated *in vitro* evidence showing that HGF-MET system may elicit cancer invasion via a paracrine loop of stroma-carcinoma interaction. This phenomenon is also demonstrated *in vivo*: anti-HGF antibody potently suppressed the tumor invasion in a mouse model of pancreas cancer (Tomioala *et al.*, 2001). On the other hand, several investigators proposed, in the late-1990's, a new concept that tumor angiogenesis inhibition leads to the arrest of cancer growth and metastasis (Yancopoulos *et al.*, 1998). Inhibition of tumor angiogenesis leads to local hypoxia, and then apoptotic death of cancer cells is associated with the arrests of tumor growth and metastasis (*i.e.*, cytostatic therapy). In this regard, NK4 also elicits an anti-angiogenic effect via perlecan-dependent mechanism. Thus, bi-functional properties of NK4 as an HGF antagonist and angiogenesis inhibitor raise a possibility that NK4 may prove therapeutic for cancer patients, as follows.

5. Anti-cancer therapy using NK4 in animal models

Carcinoma and sarcoma show malignant phenotypes prompted by a stroma-derived HGF-MET signal at least *in vitro*. If NK4 could block MET signaling as an HGF-antagonist *in vivo*, supplemental therapy with NK4 would be a pathogenesis-based strategy to counteract

tumor invasion and metastasis. This hypothesis is widely demonstrated through extensive studies using tumor-bearing animals, as described below.

5.1 First evidence of NK4 for inhibition of carcinoma progression *in vivo*

HGF, or co-cultured fibroblasts, are known to induce invasion of gallbladder carcinoma cells (GB-b1) across Matri-gel basement membrane components (Li *et al.*, 1998). NK4 competitively inhibits the binding of HGF to MET on GB-d1 cells. As a result, NK4 diminishes HGF-induced, or fibroblast-induced, motogenic activities (Date *et al.*, 1998), thus suggesting that stroma-derived HGF is a key conductor for provoking tumor invasion. Such an important role of HGF was also demonstrated *in vivo*. Subcutaneous inoculations of human gallbladder carcinoma GB-d1 cells in nude mice allow for primary tumor growth and invasion to adjacent muscular tissues. Using this conceptual model, we provided the first evidence of NK4 as an anti-tumor drug (Date *et al.*, 1998). Recombinant NK4 has inhibited the growth and muscular invasion in a mouse model of gallbladder carcinoma. Consistent with tumor growth arrest, apoptotic change becomes evident during NK4 injections. Since HGF has an anti-apoptotic effect on cancer cells (Zeng *et al.*, 2002), reverse of HGF-induced protection by NK4 may be one of the mechanisms whereby carcinoma growth can be suppressed during NK4 supplemental therapy.

5.2 Inhibition of tumor angiogenesis by NK4 treatment

In a culture of EC, NK4 produces anti-angiogenic effects via a MET-independent pathway (Kuba *et al.*, 2000; Nakabayashi *et al.*, 2003). These effects are also observed in animal models of malignant tumors: administration of recombinant NK4 suppressed primary tumor growth, metastasis of Lewis lung carcinoma, and Jyg-MC(A) mammary carcinoma implanted into mice (Kuba *et al.*, 2000), 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 in apoptotic tumor cells in primary tumors, suggesting that the inhibition of tumor growth by NK4 may be achieved by the suppression of tumor angiogenesis (Kuba *et al.*, 2000). The anti-angiogenic effects of NK4 are widely demonstrated in various types of cancers [see our review articles (Matsumoto & Nakamura, 2005; Matsumoto *et al.*, 2008a,b)]. Because the inhibition of angiogenesis by NK4 leads to tumor hypoxia, hypoxia-primed apoptosis may contribute to a reduction in tumor size during NK4 supplemental therapy.

5.3 Delayed NK4 therapy for attenuation of end-stage pancreas carcinoma

Anti-tumor effect of NK4 is also observed in a mouse model of advanced pancreas carcinoma (Tomioka *et al.*, 2001). When NK4 treatment was initiated on day 10, a time when cancer cells were already invading surrounding tissues, NK4 potently inhibited the tumor growth, peritoneal dissemination, and ascites accumulation at 4 weeks after the inoculation. Such an anti-tumor effects of NK4 correlated with decreased vessel density in pancreatic tumors. In an end-stage of pancreas cancer, NK4 inhibited the malignant phenotypes, such as peritoneal dissemination, invasion of cancer cells into the peritoneal walls and ascites accumulation (Tomioka *et al.*, 2001). As a result, NK4 prolonged the survival time of mice at an end-stage of cancer (**Fig. 4**). Because effective systemic therapy for pancreatic cancer is currently not available, and diagnosing pancreatic cancer in its early stages is difficult, the highly invasive and metastatic behaviors of pancreatic cancer lead to difficulty in attaining a

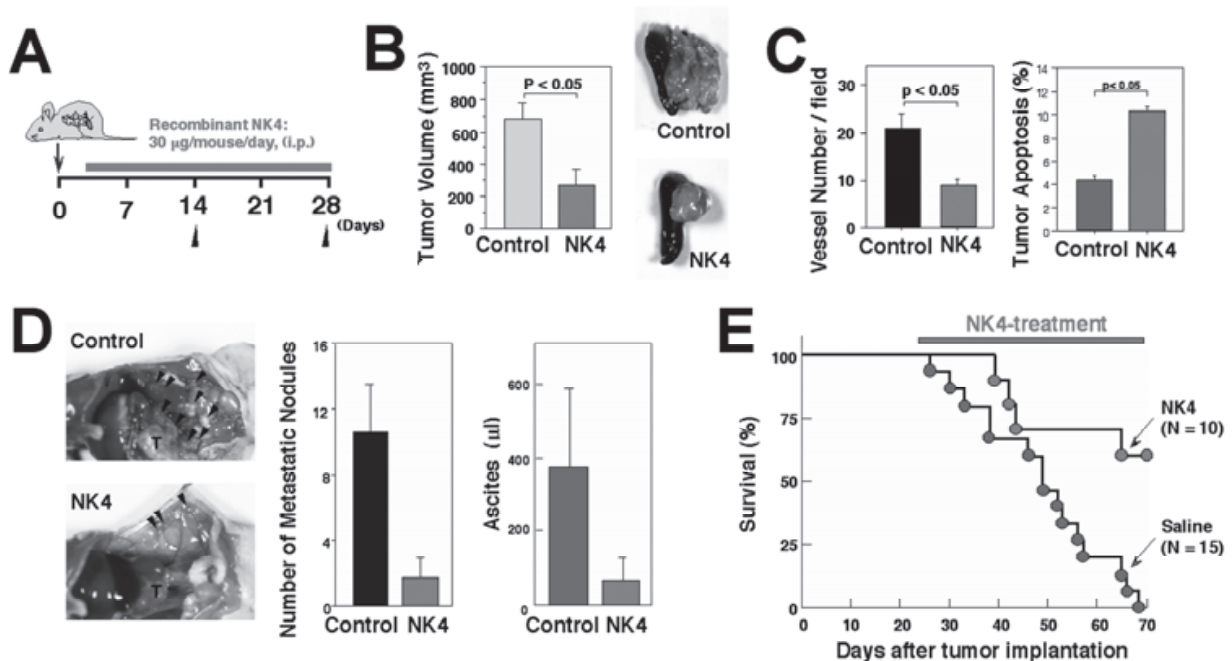


Fig. 4. Anti-tumor effects of NK4 on advanced pancreas cancer in mice. (A) Schedules for NK4 treatment of mice with pancreatic cancer. NK4 was injected into mice between 3 and 28 days after the inoculation of human pancreatic cancer cells (SUIT-2). (B) Inhibition of primary tumor growth by NK4. Photographs show appearance of the primary pancreatic cancer. (C) Histological analysis of the effect of NK4-treatment on tumor angiogenesis (left) and apoptosis (right). NK4-treatment reduced the number of vessel numbers, while apoptotic death of cancers was enhanced by NK4. (D) Inhibitory effects of NK4 on peritoneal metastasis. Left: Typical macroscopic findings. Middle: Changes in the number of metastatic nodules. Right: Changes in the ascite volumes. (E) Prolonged survival of tumor-bearing mice treated with NK4.

long-term survival and a recurrence-free status. Targeting tumor angiogenesis and blockade of HGF-mediated invasion of cancer cells may prove to be potential therapy for patients with pancreatic cancer.

5.4 Therapy combining NK4 with other treatments

Anti-cancer chemotherapy is widely used for the suppression of malignant tumors with or without surgical treatment. Therapy regimens that combine anti-cancer chemo drugs and NK4 enhance their anti-tumor effect (Matsumoto *et al.*, 2011). Irradiation therapy often enhances cancer metastasis, especially in cases of pancreatic carcinoma, and this is associated with the irradiation-induced up-regulation of HGF in fibroblasts (Qian *et al.*, 2003; Ohuchida *et al.*, 2004). Thus, NK4 may overcome these irradiation-associated side effects.

Epidermal growth factor receptor (EGFR) kinase inhibitors, such as Gefitinib, are used to treat non-small cell lung cancers that have activating mutations in the EGFR gene, but most of these tumors become resistant to EGFR-kinase inhibitors due to enhancement of HGF-MET signals (Engelman *et al.*, 2007; Yano *et al.*, 2008; Okamoto *et al.*, 2010). Thus, NK4 treatment may reverse HGF-induced resistance to Gefitinib.

Recently, it was demonstrated that NK4-mediated tumor regression depends on the infiltration of cytotoxic T lymphocytes (Kubota *et al.*, 2009b). Importantly, depletion of CD8+ cells markedly abrogated the anti-tumor activity of NK4 in a mouse model of colon cancer. NK4 enhances immune responses in dendritic cells *in vitro*. Thus, NK4 may also have utility for anti-tumor immunotherapy.

There is now ample evidence that NK4 is useful for the inhibition of growth, invasion and metastasis in various types of tumors, such as gastric carcinoma (Hirao *et al.*, 2002), pancreas cancer (Tomioka *et al.*, 2001), prostate cancer (Davies *et al.*, 2003), multiple myeloma (Du *et al.*, 2007) and melanoma (Kishi *et al.*, 2009) (**Table-1**). These results support our hypothesis that HGF is a key determinant of tumor malignancy (Matsumoto *et al.*, 1996b).

Tumor diseases (Cell lines and treatment)	NK4 therapy	Outcome	Literature
A. Digestive system:			
Gastric carcinoma (TMK1 cells, ip, Mouse)	Adeno-NK4, ip	Inhibitions of growth and metastasis, Anti-angiogenesis, Reduced ascites	Ueda K <i>et al.</i> , Eur J Cancer 40: 2135-2142 (2004)
Hepatic carcinoma (HUH7 cells, portal vein, Mouse)	Adeno-NK4, iv	Inhibitions of growth, Anti-angiogenesis, Prolonged survival	Son G <i>et al.</i> , J Hepatol 45: 688-695 (2006)
Gallbladder cancer (GB-d1 cells, sc, Mouse)	NK4, sc	Inhibitions of growth and invasion	Date K <i>et al.</i> , Oncogene 17: 3045-354 (1998)
Pancreatic carcinoma (SUIT-2 cells, intra-pancreas, Mouse)	r-NK4, ip	Inhibitions of growth, invasion and metastasis, Anti-angiogenesis, Reduced ascites, Prolonged survival	Tomioka Det <i>et al.</i> , Cancer Res 61: 7518-7524 (2001)
Colon carcinoma (MC-38 cells, intra-spleen, Mouse)	NK4 cDNA, bolus iv (hydrodynamics)	Inhibitions of growth, invasion and metastasis, Anti-angiogenesis, Prolonged survival	Wen J <i>et al.</i> , Cancer Gen Ther 11: 419-430 (2004)
B. Respiratory system:			
Lung carcinoma (Lewis carcinoma, sc, Mouse)	r-NK4, sc	Inhibitions of growth and metastasis, Anti-angiogenesis, Enhanced apoptosis	Kuba K <i>et al.</i> , Cancer Res 60: 6737-6743 (2000)
Lung carcinoma (A549 cells, sc, Mouse)	Adeno-NK4, intra-tumor or ip	Inhibition of growth, Anti-angiogenesis	Maemondo M <i>et al.</i> , Mol Ther 5: 177-185 (2002)
Mesothelioma (EHMES-10 cells, sc, Mouse)	Adeno-NK4, intra-tumor	Inhibition of growth, Enhanced apoptosis, Anti-angiogenesis	Suzuki Y <i>et al.</i> , Int J Cancer 127: 1948-1957 (2010)

C. Reproductive system:			
Prostate carcinoma (PC-3 cells, sc, Mouse)	r-NK4, sc (osmotic pump)	Inhibition of growth, Anti-angiogenesis	Davies G <i>et al.</i> , Int J Cancer 106: 348-354 (2003)
Ovarian carcinoma (HRA cells, ip, Mouse)	NK4 gene, Stable transfection	Inhibition of metastasis, Prolonged survival	Saga Y <i>et al.</i> , Gene Ther 8: 1450-1455 (2001)
D. Hematopoietic system:			
Lymphoma (E.G7-OVA cells, sc, Mouse)	Adeno-NK4, intra-tumor (with DC)	Inhibition of growth, Anti-angiogenesis, Induction of CTL	Kikuchi T <i>et al.</i> , Blood 100: 3950-3959 (2003)
Multiple myeloma (KMS11/34 cells, sc, Mouse)	Adeno-NK4, im	Inhibition of growth, Anti-angiogenesis, Enhanced apoptosis	Du W <i>et al.</i> , Blood 109: 3042-3049 (2007)
E. Other organ or tissues:			
Melanoma (B16F10 cells, sc, Mouse)	Adeno-NK4, iv	Inhibitions of growth and metastasis, Anti-angiogenesis	Kishi Y <i>et al.</i> , Cancer Sci 100: 1351-1358 (2009)
Glioblastoma (U-87 MG cells, Intra-brain, Mouse)	r-NK4, intra-tumor	Inhibition of growth, Anti-angiogenesis, Enhanced apoptosis	Brockmann MA <i>et al.</i> , Clin Cancer Res 9: 4578-4585 (2003)
Breast carcinoma (MDAMB231 cells, sc, Mouse)	r-NK4, sc	Inhibition of growth, Anti-angiogenesis	Martin TA <i>et al.</i> , Carcinogenesis 24: 1317-1323 (2003)

Adeno-NK4, adenoviral vector carrying NK4 cDNA; r-NK4, recombinant NK4 protein; sc, subcutaneous; iv, intravenous; ip, intraperitoneal; im, intramuscular; DC, dendritic cells; and CTL, cytotoxic T lymphocytes.

Table 1. Representative studies to show therapeutic effects of NK4 on distinct types of tumors in animal models

6. Hydrodynamics-based NK4 gene therapy for colon cancer inhibition

Hydrodynamic delivery has emerged as the simplest and effective method for intracellular delivery of subjective genes in rodents; this process requires no special equipment. The system employs a physical force generated by the rapid injection of a large volume of solution into a blood vessel to enhance the permeability of endothelium and the plasma membrane of the parenchyma cells, such as hepatocytes, to facilitate a delivery of the substance into cells (Bonamassa *et al.*, 2011). Using this technique in mice, we established an endocrine delivery system for NK4 that leads to an inhibition of the malignant behavior of cancers, as follows.

6.1 NK4 supplementation system via hydrodynamic gene delivery in mice

Numerous clinical studies have indicated the apparent increases in serum HGF levels in patients during the progression of cancers (Wu *et al.*, 1998; Osada *et al.*, 2008). It is likely that HGF in blood protects cancer cell suspension from anoikis-like cell death (Zeng *et al.*, 2002). Thus, we predict that over-production of NK4 in blood would overcome the HGF-mediated metastatic events seen in blood flow (and possibly in local sites). Hydrodynamic-based gene delivery is known to achieve an efficient expression of exogenous genes predominantly in the liver but much lesser in the kidney and spleen (Suda *et al.*, 2007). Based on this background, we established a method for the induction and maintenance of higher levels of NK4 in blood through repeated injections of NK4 cDNA-containing plasmid.

For hydrodynamic-based gene delivery, 5 microgram of plasmid DNA (pCAGGS-NK4), or pCAGGS-empty (as a control), in saline was injected within 5 seconds into tail veins of mice at 2.4 ml per 30g body weight (Wen *et al.*, 2004; 2007). As a result, exogenous NK4 was detected, and plasma NK4 reached a mean value of 49.5 ng/ml 24 hours post-bolus injection and decreased to 15.4 ng/ml on day 3. Following the second and third injections, the plasma NK4 level again reached approximately 70 and 130 ng/ml on days 8 and 15, respectively. Thus, plasma NK4 levels increased following additional administration of the expression plasmid, and were maintained at levels of > 8 ng/ml during 3 weeks post-treatment (Fig. 5).

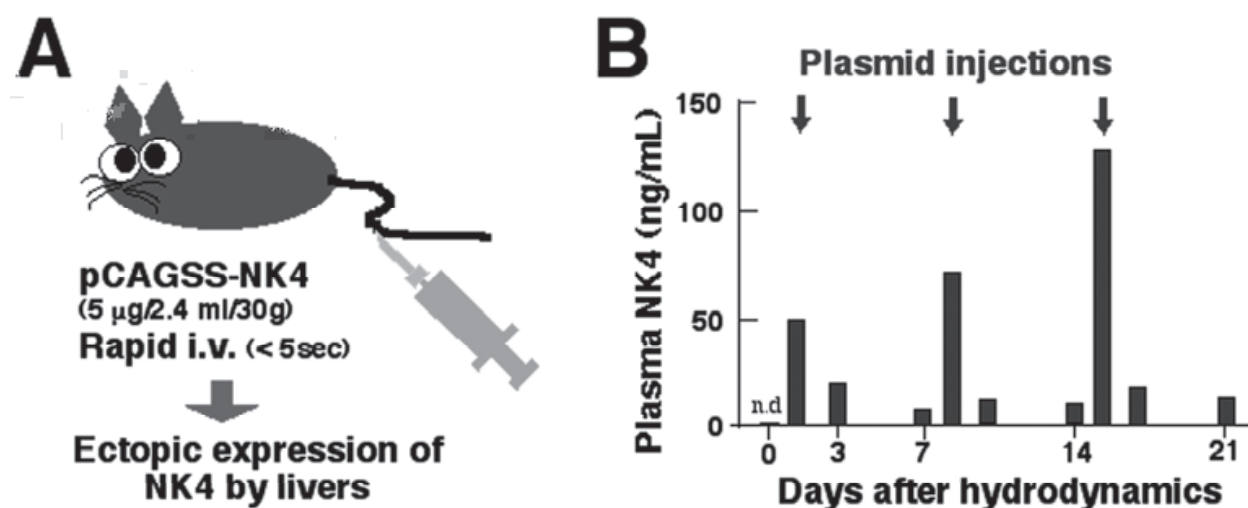


Fig. 5. Hydrodynamics-mediated NK4 delivery system in mice. (A) An experimental protocol of NK4 gene administration. Five microgram of pCAGGS-NK4 was administered intravenously into mice on day 0, 7 and 14. (B) Changes in plasma NK4 levels following repetitive administration of expression plasmid for NK4. Arrows mean the time of plasmid administration. See reference (Wen *et al.*, 2007) for further information.

6.2 Inhibition of colon cancer metastasis by NK4 gene delivery

Colon cancer is one of the most common cancers in the world, with a high propensity to metastasize: 30-40% of patients have metastatic disease at the initial diagnosis. The liver is the most frequent site of metastasis, and hepatic failure is a lethal event during colon cancer. Thus, direct inhibition of the dissociation, spreading and invasion of cancer cells is expected to become efficient treatment. With regard to this, HGF stimulates the invasion of MC-38 mouse colon cancer cells across MatriGel (Parr *et al.*, 2000), which is composed of laminin and other matrices and mimics the basement membrane *in vivo*. In this model, NK4 has

inhibited the HGF-mediated migration of MC-38 cells in a culture model of colon cancer invasion. This anti-invasive effect of NK4, obtained by *in vitro* studies, is demonstrated *in vivo* in the following two studies.

An hepatic metastatic model was prepared by the injection of mouse MC-38 cells into the spleen. During the progression of colon cancer in hepatic tissues, HGF was over-produced by hepatic sinusoidal cells, while MET tyrosine phosphorylation became evident, particularly around the front lines of invasive zones. Supplementation of NK4 in blood and livers via a single injection of NK4-cDNA containing plasmid (pCAGGS-NK4) resulted in the loss of MET tyrosine phosphorylation (Fig. 6). Under such a MET-inactivated condition by NK4 treatment, hepatic invasion by colon carcinoma was strongly inhibited (Wen *et al.*, 2004).

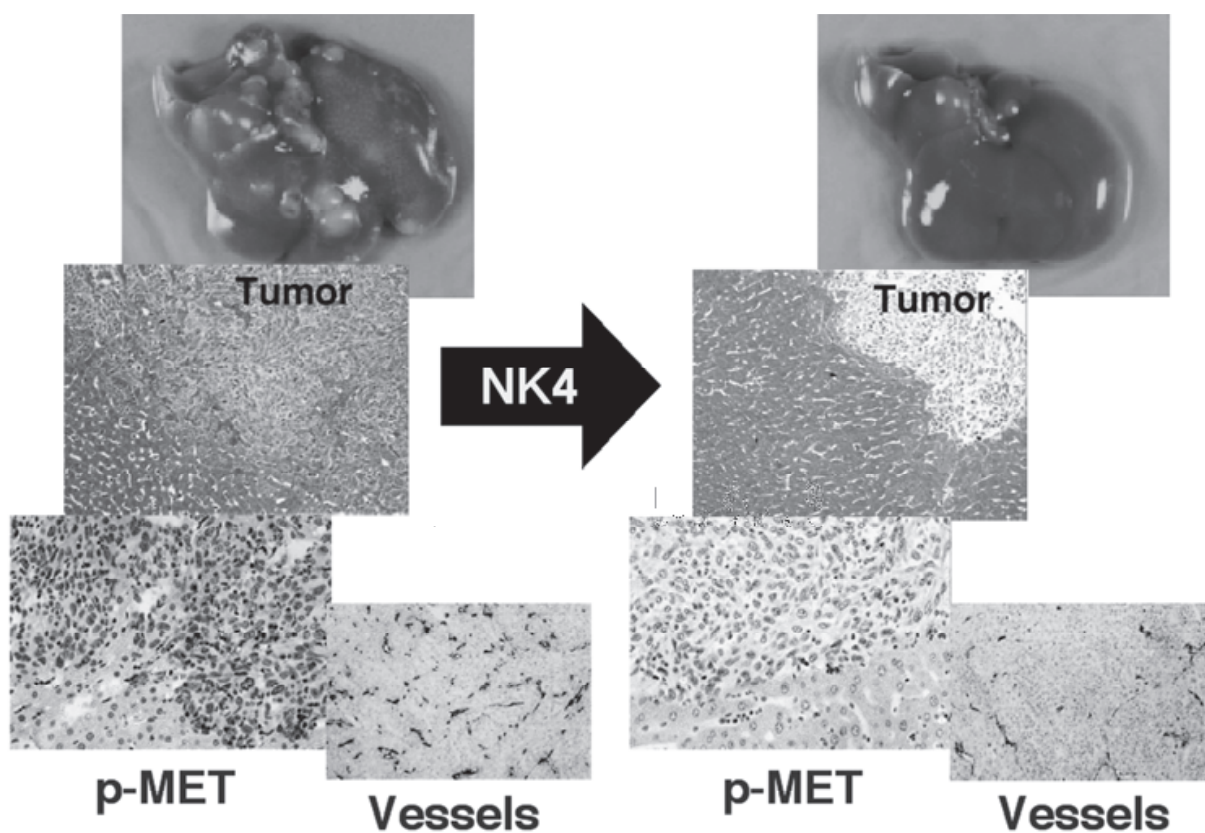


Fig. 6. Successful outcome of hydrodynamics-based NK4 gene therapy in a mouse model of colon cancer. The hepatic invasion model is prepared by intra-splenic inoculation of MC-38 colon carcinoma in mice. In the control group, invasion of carcinoma cells into neighboring hepatic areas becomes evident, along with an induction of MET tyrosine phosphorylation (p-MET) and an increase in vessel numbers. In contrast, NK4 suppresses tumor invasion by inhibiting MET tyrosine phosphorylation and reducing angiogenesis. As a result, NK4 gene therapy prolongs the survival of these mice (Wen *et al.*, 2004).

Repeated administrations of NK4-containing plasmid DNA also inhibited the malignant behaviors of colon carcinoma (Wen *et al.*, 2007). Actually, NK4 repetitive gene therapy potently inhibited the muscular invasion of MC-38 carcinoma cells. Furthermore, angiogenesis in the colon cancer was markedly suppressed by NK4 repetitive therapy, along with an increase in tumor apoptosis. Overall, the number of hepatic metastatic nodules was

dramatically decreased by the repeated injections of NK4-cDNA containing plasmid. This study provides an anti-tumor model where NK4 is supplemented via a hydrodynamics-based gene therapy (Wen *et al.*, 2007).

Recently, hydrodynamic gene delivery using a rapid injection of a relatively large volume of DNA solution has facilitated experimental gene therapy studies, particularly in rodents (Suda *et al.*, 2007). This method is superior to the existing delivery systems because of its simplicity, efficiency, and versatility. Hydrodynamic gene delivery is also useful for supplementation of HGF, an intrinsic repair factor, for the inhibition of, or recovery from, intractable organ diseases, such as acute renal failure (Dai *et al.*, 2002) or pulmonary airway hyper-responsiveness during asthma (Okunishi *et al.*, 2005). In these experiments, plasma HGF levels were sustained within a pharmacological range (3-30 ng/ml). Wide success in applying hydrodynamic principles to delivery of NK4- or HGF-related DNA, RNA, proteins, and synthetic compounds, into the cells in various tissues of small animals, has inspired the recent attempts at establishing a hydrodynamic procedure for clinical use.

7. Summary and perspective

NK4-related studies provided a proof-of-concept that MET signaling from stroma-derived HGF plays a pivotal role in eliciting tumor invasion and metastasis (Matsumoto & Nakamura, 2005; Nakamura *et al.*, 2010). Human genetic studies also strengthened the important role of MET activation for tumor malignancy. There is now ample evidence to demonstrate the role of MET mutations in tumor malignancy (Lengyel *et al.*, 2007; Matsumoto *et al.*, 2008a,b; Pao *et al.*, 2011). Of interest, mutation of the von-Hippel-Lindau (VHL) gene leads to renal clear cell carcinoma through constitutive MET tyrosine phosphorylation (Nakaigawa *et al.*, 2006), hence suggesting a critical role of wild-type VHL in inhibiting MET over-activation as a negative regulator.

During the progression of malignant tumors, soluble MET is producible by carcinoma cells through an ectodomain shedding cascade (Wader *et al.*, 2011). Soluble MET inhibits the HGF-MET complex and signaling transduction. Thus, MET shedding system is considered as a self-defense response that minimizes tumor metastasis. Likewise, an NK4-like fragment of the HGF α -chain can be secreted from human breast carcinoma, which inhibits MET tyrosine phosphorylation (Wright *et al.*, 2009). Thus, "endogenous" soluble MET and NK4-like variant appear to reduce HGF-MET signaling and delay tumor progression, but this response is insufficient, allowing for tumor metastasis. Thus, supplemental therapy with NK4 is a reasonable strategy to completely block tumor metastasis.

The hope is that angiogenesis inhibition might control tumor metastasis (Yancopoulos *et al.*, 1998). However, long-term use of angiogenesis inhibitors, such as VEGF inhibitor, results in hypoxia-resistance (Fischer *et al.*, 2007), possibly due to hypoxia-induced MET up-regulation by cancer (Bottaro & Liotta, 2003). NK4 is an angiogenesis inhibitor with the ability to inhibit MET activation, and discovery of this fragment opened up a new avenue for the development of freeze-and-dormancy therapy (Fig. 7). Thus, NK4 is now defined as "Malignostatin". In addition to NK4, several anti-metastatic drugs have been proposed, with a major focus on small molecules that inhibit the tyrosine kinase activity of MET; ribozyme; small-interfering RNA; anti-HGF antibodies; soluble MET; and HGF-variant decoys (Jiang *et al.*, 2005; Benvenuti & Comoglio, 2007; Eder *et al.*, 2009; Underiner *et al.*, 2010; Cecchi *et al.*, 2010). HGF-MET targeting research will shed more light on cancer biology, pathology and new technologies to overcome host death due to cancer metastasis.

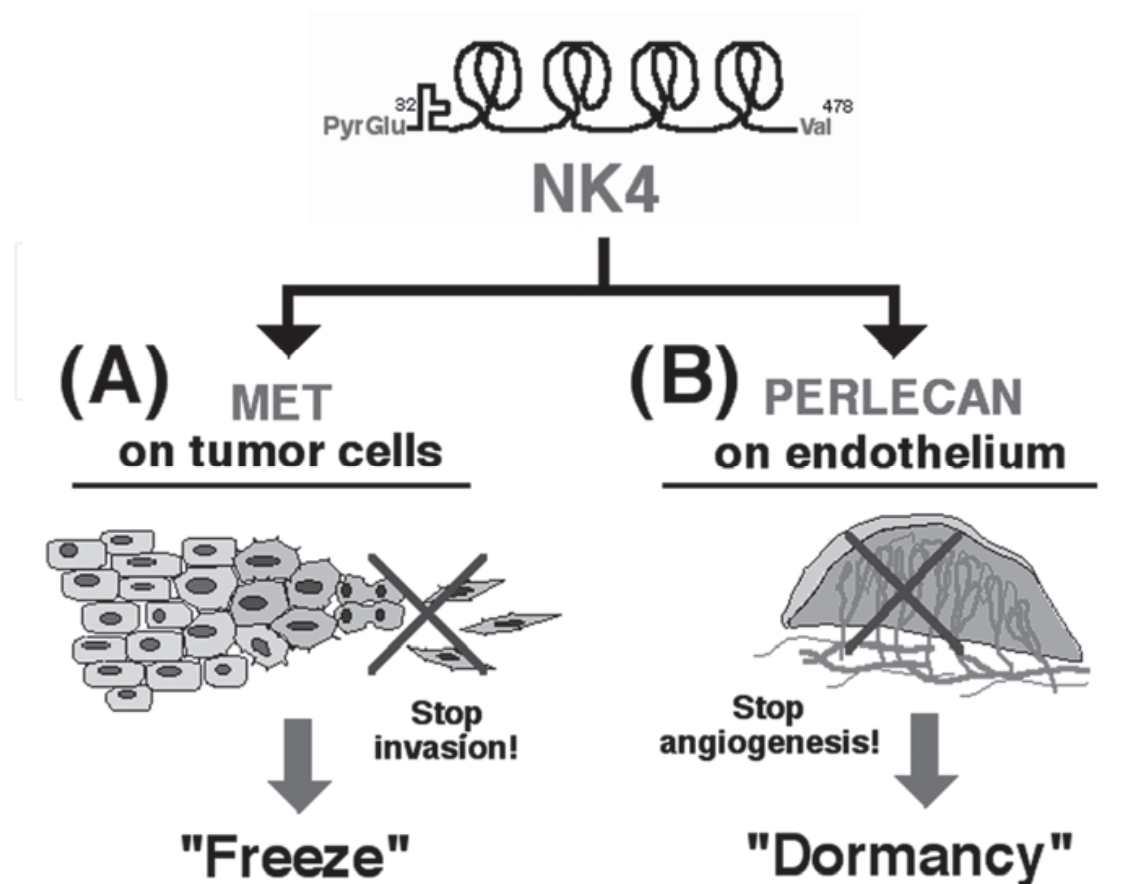


Fig. 7. Freeze-and-dormancy therapy of malignant tumors by NK4/malignostatin. NK4 blocks tumor invasion and metastasis through an inhibition of HGF-MET signals as an HGF-antagonist. Furthermore, NK4 inhibits tumor angiogenesis via a perlecan-dependent mechanism. Such a dual function of NK4 contributes to "freeze" and "dormancy" anti-cancer therapy.

8. Acknowledgement

The publication of this manuscript was supported by grants from the Ministry of Education, Science, Technology, Sports and Culture of Japan (the 21st Century global COE program to TN). We would like to thank all co-workers for the continuous studies on the roles of HGF and NK4 during cancer progression. We are also grateful to James L. McDonald (Scientific Editorial Services; Harrison, AR) for language assistance.

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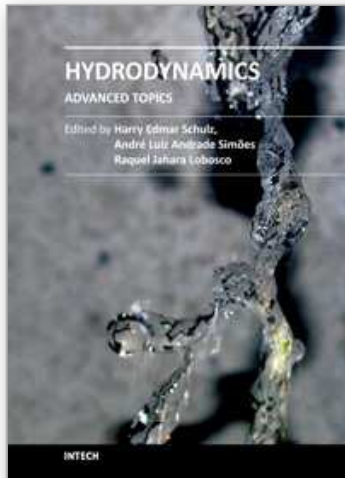
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Hydrodynamics - Advanced Topics

Edited by Prof. Harry Schulz

ISBN 978-953-307-596-9

Hard cover, 442 pages

Publisher InTech

Published online 22, December, 2011

Published in print edition December, 2011

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Shinya Mizuno and Toshikazu Nakamura (2011). Endocrine Delivery System of NK4, an HGF-Antagonist and Anti-Angiogenic Regulator, for Inhibitions of Tumor Growth, Invasion and Metastasis, Hydrodynamics - Advanced Topics, Prof. Harry Schulz (Ed.), ISBN: 978-953-307-596-9, InTech, Available from: <http://www.intechopen.com/books/hydrodynamics-advanced-topics/endocrine-delivery-system-of-nk4-an-hgf-antagonist-and-anti-angiogenic-regulator-for-inhibitions-of->

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