Targeting the tumor and its microenvironment by a dual-function decoy Met receptor

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Summary

Met, the receptor for hepatocyte growth factor (HGF), is activated in human cancer by both ligand-dependent and -independent mechanisms. We engineered a soluble Met receptor (decoy Met) that interferes with both HGF binding to Met and Met homodimerization. By lentiviral vector technology, we achieved local or systemic delivery of decoy Met in mice. We provide evidence that in vivo expression of decoy Met (1) inhibits tumor cell proliferation and survival in a variety of human xenografts, (2) impairs tumor angiogenesis by preventing host vessel arborization, (3) suppresses or prevents the formation of spontaneous metastases, and (4) synergizes with radiotherapy in inducing tumor regression, without (5) affecting housekeeping physiological functions in the adult animal.

Introduction

Hepatocyte growth factor (HGF, also known as scatter factor) is a pleiotropic cytokine of mesenchymal origin that controls cell proliferation, survival, motility, and differentiation in a variety of tissues, including epithelial, endothelial, neuronal, and hemopoietic cells (Rubin et al., 1993; Zarnegar and Michalopoulos, 1995; Tamagnone and Comoglio, 1997). The coordinated orchestration of these biological processes by HGF results in a specific genetic program known as "invasive growth" (reviewed by Trusolino and Comoglio, 2002). This complex program has evolved primarily to master vital morphogenetic processes during embryo development and organ formation (Woolf et al., 1995; Takayama et al., 1996; Andermarcher et al., 1996). Experimental deletion of the *hgf* gene—or of the gene encoding its high affinity receptor, the tyrosine kinase Met—results in placental and hepatic abnormalities leading to in utero embryo death (Schmidt et al., 1995; Uehara et al., 1995; Bladt et al., 1995).

In the adult organism, the physiologic role of the HGF pathway is less well understood, although several lines of evidence suggest that it may be involved in wound healing, tissue regeneration, hemopoiesis, and tissue homeostasis in general (Miyazawa et al., 1994; Yanagita et al., 1993; Galimi et al., 1994; Matsumoto and Nakamura, 1997). It is, however, clear that HGF/Met signaling is exploited by tumors to grow, survive, and expand, mimicking developing organs or healing wounds (Birchmeier et al., 2003; Comoglio and Trusolino, 2002; Vande Woude et al., 1997).

While the Met receptor is expressed by most tissues of epithelial, hemopoietic, and neuronal origin, HGF is secreted by mesenchymal cells, but it accumulates ubiquitously in tissues due to its high affinity for extracellular matrix (Kobayashi et al., 1994; Lyon et al., 1994). Met overexpression is a common event in human cancer (reviewed by Trusolino and Comoglio, 2002), and may determine ligand-independent receptor activation (Wang et al., 2001) or increased sensitivity to environmental HGF (Pennacchietti et al., 2003).

Various approaches have been explored to inhibit HGF or Met in experimental systems, including neutralization of HGF by monoclonal antibodies (Cao et al., 2001), ribozyme-mediated downregulation of HGF/Met expression (Jiang et al., 2001, 2003; Abounader et al., 2002; Herynk et al., 2003; Kim et al., 2003), impairment of receptor dimerization by dominant-negative Met (Firon et al., 2000; Furge et al., 2001), inhibition of Met tyrosine

SIGNIFICANCE

Tyrosine kinase inhibitors and antiangiogenic agents have recently shown great promise in cancer therapy. However, involvement of tyrosine kinase pathways in important physiologic processes has raised concerns about the toxicity of tyrosine kinase inhibitors. Similarly, pure antiangiogenic agents have proved less effective than anticipated in early clinical trials, suggesting that simultaneous targeting of host vessels and cancer cells may be more effective than antiangiogenic therapy alone. Here, we show that inhibition of a single tyrosine kinase receptor achieves concomitant suppression of cancer cell proliferation, survival, and invasion, and of host angiogenesis, without interfering with normal physiologic functions. These data point to Met as an ideal target for antineoplastic therapy, and provide proof-of-concept for further clinical testing of HGF/Met antagonists.

kinase activity by small molecule inhibitors (Sattler et al., 2003; Christensen et al., 2003; Wang et al., 2003), and ligand displacement by a competitive inhibitor of HGF (reviewed by Matsumoto and Nakamura, 2003).

In the present study, we employed an alternative approach based on (1) a soluble Met receptor (Michieli et al., 1999) and (2) an improved lentiviral vector technology (Follenzi et al., 2000). The data presented here not only point to Met as an ideal target for cancer therapy, but also suggest that the extracellular portion of the Met receptor is a promising therapeutic agent by itself.

Results

Engineering of decoy Met

To generate a soluble HGF receptor, we engineered a recombinant protein corresponding to the entire extracellular domain of Met, truncated before the transmembrane domain (Figure 1A). As a control, we also engineered a truncated form of HGF, known as NK4 HGF, which has been shown to compete with HGF in Met binding (Matsumoto and Nakamura, 2003). A Myc-epitope tag and a poly-histidine tag were added at the C terminus of both molecules. The cDNAs for the engineered molecules were subcloned into the pRRLsin.PPT.CMV.Wpre lentiviral vector (Follenzi et al., 2000; Supplemental Figure S1 at http://www.cancercell.org/cgi/content/full/6/1/61/DC1). Into the same vector, we also subcloned the cDNA for human HGF. Recombinant viral particles from recombinant vectors were produced in large scale and used to transduce a panel of human tumor cell lines (MDA-MB-435 and MDA-MB-435-β4, human mammary carcinoma; A549, human lung carcinoma) and primary human umbilical vein endothelial cells (HUVECs). The different recombinant factors reached comparable concentrations in the conditioned medium of transduced cells, approximately 30–60 nM in 72 hr (Supplemental Figure S2).

Decoy Met binds at high affinity to both HGF and full-size Met

Starting from the conditioned medium of lentiviral vector-transduced tumor cells, NK4 HGF and decoy Met were purified to homogeneity by affinity chromatography (Figure 1B), and their ability to bind Met or HGF, respectively, was analyzed by ELISA (Figure 1C). Purified Fc-Met chimera was used as a control for HGF binding (Mark et al., 1992). NK4 HGF bound Met with approximately one log less affinity compared to HGF (HGF, K_D = 0.035 nM; NK4 HGF, K_D = 0.326 nM). In contrast, human HGF bound decoy Met with a K_D of 0.049 nM, a value which corresponds approximately to the in vivo affinity of HGF for bona fide Met (Matsumoto et al., 1998).

Since the extracellular portion of Met is conceivably involved in receptor homodimerization, we also tested the ability of decoy Met to interact with full-size Met in (1) coimmunoprecipitation experiments and (2) ELISA binding assays. In lentiviral vector-transduced MDA-MB-435 cells, decoy Met (but not NK4 HGF) coimmunoprecipitated with endogenous Met, and vice versa (Supplemental Figure S3). In ELISA binding assays, purified decoy Met bound Fc-Met at high affinity (Figure 1D), following a biphasic curve (first site, K_D = 0.058 nM; second site, K_D = 2.840 nM). Consistent with this, we observed that two distinct isolated subdomains of decoy Met, the SEMA domain and the IPT region, conserve the ability of coimmunoprecipitating with full-size Met (not shown). Thus, decoy Met interacts with high affinity with both HGF and full-size Met.

Decoy Met inhibits Met activation mediated by HGF or by ligand-independent mechanisms

To test whether the engineered antagonists could inhibit ligand-induced Met activation, we stimulated lentiviral vector-transduced cells with recombinant HGF, and then determined the extent of Met tyrosine phosphorylation. In MDA-MB-435 cells, cells transduced with NK4 HGF or decoy Met lentivirus showed markedly reduced sensitivity to HGF stimulation compared to cells transduced with control lentivirus (Figure 1E, MDA panel). Similar results were obtained with A549 cells and HUVECs, thus suggesting that both NK4 HGF and decoy Met prevent HGF-induced Met activation in a variety of cellular models. However, Met is often activated in cancer by HGF-independent mechanisms (reviewed by Trusolino and Comoglio, 2002). We therefore tested whether NK4 HGF or decoy Met could inhibit Met activation induced by (1) overexpression or (2) oncogenic point mutation in different cell systems.

In COS cells, transient overexpression of exogenous Met results in spontaneous dimerization and autoactivation due to very high protein levels. In this model, coexpression of decoy Met—but not NK4 HGF—significantly reduced wild-type Met phosphorylation (Figure 1E, COS panel). In GTL16 gastric carcinoma cells, Met is constitutively activated due to natural receptor overexpression (Giordano et al., 1989). In this system too, expression of decoy Met dramatically reduced constitutive Met tyrosine phosphorylation, while NK4 HGF was not effective (Figure 1E, GTL16 panel).

In hereditary and sporadic papillary carcinomas of the kidney, in sporadic hepatocellular carcinomas, and in head and neck primary tumors and metastases, Met has been found to be activated by point mutation (Schmidt et al., 1997; Park et al., 1999; Di Renzo et al., 2000). To test whether NK4 HGF or decoy Met could interfere with point mutation-induced Met activation, we transduced TOV-112D human ovarian carcinoma cells (which lack Met expression; see below) with a lentiviral vector encoding wild-type or mutated Met, or an empty vector as control. Cells overexpressing mutant Met (M1250T) displayed constitutive tyrosine phosphorylation (Figure 1E, TOV panel). These cells were further transduced with empty, NK4 HGF, or decoy Met vector, and the extent of Met tyrosine phosphorylation was determined by immunoblotting. Once again, decoy Met could efficiently prevent point mutation-induced Met activation, while NK4 HGF was not effective (Figure 1E, TOV panel). Therefore, consistent with the biochemical data presented above, decoy Met is a soluble HGF antagonist capable of interfering with both HGF-induced and ligand-independent Met activation.

Decoy Met inhibits the pleiotropic effects of HGF in vitro

In a first experimental approach, we tested the ability of HGF antagonists to inhibit HGF-dependent biological activity on tumor cells in a variety of in vitro assays. We subjected lentiviral vector-transduced cells to (1) a proliferation assay, (2) a survival assay, (3) a Matrigel invasion assay, (4) a branching morphogenesis assay, and (5) a "scratch" assay, either in the absence or presence of recombinant HGF. This analysis revealed that both NK4 HGF and decoy Met—at the concentrations reached by lentiviral vector-mediated expression—effectively inhibited HGF-induced DNA synthesis (Figure 2A), protection against

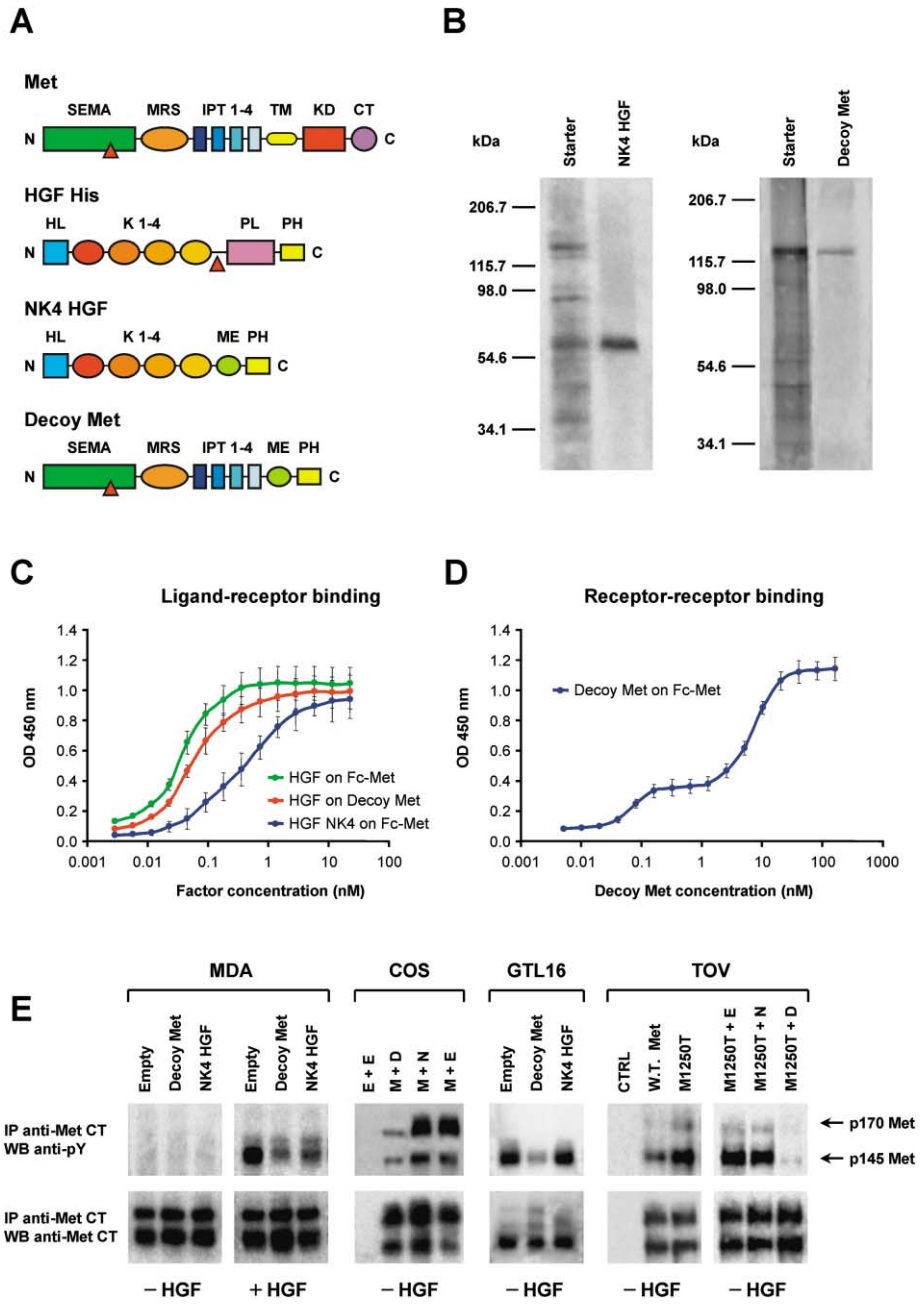

Figure 1. Engineering of a soluble Met receptor

A: Schematic representation of the recombinant proteins used in this study. SEMA, semaphorin domain; MRS, Met-related sequence; IPT 1-4, immunoglobulin-plexin-transcription factor domain 1-4; TM, trans-membrane domain; KD, kinase domain; CT, C-terminal tail; ME, Myc epitope; PH, poly-His; HL, hairpin loop; K 1-4, kringle 1-4; PL, protease-like domain. The red triangles indicate the proteolytic site dividing the α chain (left) from the β chain (right).

B: Coomassie staining of NK4 HGF and decoy Met purified by affinity chromatography and resolved by SDS-PAGE in nonreducing conditions. Starter, conditioned medium prior to purification.

C: Ligand-receptor ELISA binding assay: HGF on Fc-Met (in green); HGF on decoy Met (in red); NK4 HGF on Fc-Met (in blue).

D: Receptor-receptor ELISA binding assay: decoy Met on Fc-Met.

E: Inhibition of Met activation induced by HGF stimulation (MDA-MB-435 cells), receptor overexpression (COS and GTL16 cells), or oncogenic point mutation (TOV-112D cells). E, empty vector; M, full-size Met; D, decoy Met; N, NK4 HGF; pY, phosphotyrosine; CTRL, cells transduced with empty vector; wt, wild-type; M1250T, mutant Met bearing the M1250T amino acid substitution. The higher Met band (p170 Met) corresponds to unprocessed Met, while the lower band (p145 Met) is the mature form of Met. Different cell lines may display different patterns of p170/p145 phosphorylation.

apoptosis (Figure 2B), Matrigel invasion (Figure 2C), branching morphogenesis (Figure 2D), and scratch repair (Figure 2E), thus demonstrating that the engineered antagonists can functionally neutralize the effects of HGF on living cells. However, in Matrigel invasion assays using GTL16 gastric carcinoma cells, in which, as described above, Met is constitutively activated, only decoy Met inhibited cell migration in the absence of HGF, thus confirming that decoy Met—but not NK4 HGF—is capable of blocking receptor activation in a dominant-negative fashion (Figure 2F).

Decoy Met impairs HGF-induced endothelial cell migration and branching

Since HGF is a potent angiogenic factor (Bussolino et al., 1992), we analyzed the effect of NK4 HGF and decoy Met on endothelial cell migration and morphogenesis. Lentiviral vector-transduced HUVECs were analyzed for their ability (1) to migrate through a fibronectin layer (Figure 3A) and (2) to form capillary-like branched structures in a collagen gel (Figure 3B), in the presence of no factor, recombinant vascular endothelial growth factor (VEGF), recombinant HGF, or both VEGF and HGF. This analysis revealed that VEGF and HGF cooperate in inducing endothelial cell motility and particularly in promoting capillary branching (see Supplemental Figure S4 at http://www.cancercell.org/cgi/content/full/6/1/61/DC1 for representative images). In both assays, decoy Met inhibited invasion mediated by HGF but not by VEGF, indicating that its activity is specific for the HGF/Met pathway. In contrast, as previously reported (Kuba et al., 2000), NK4 HGF inhibited equally well HGF- or VEGF-medi-

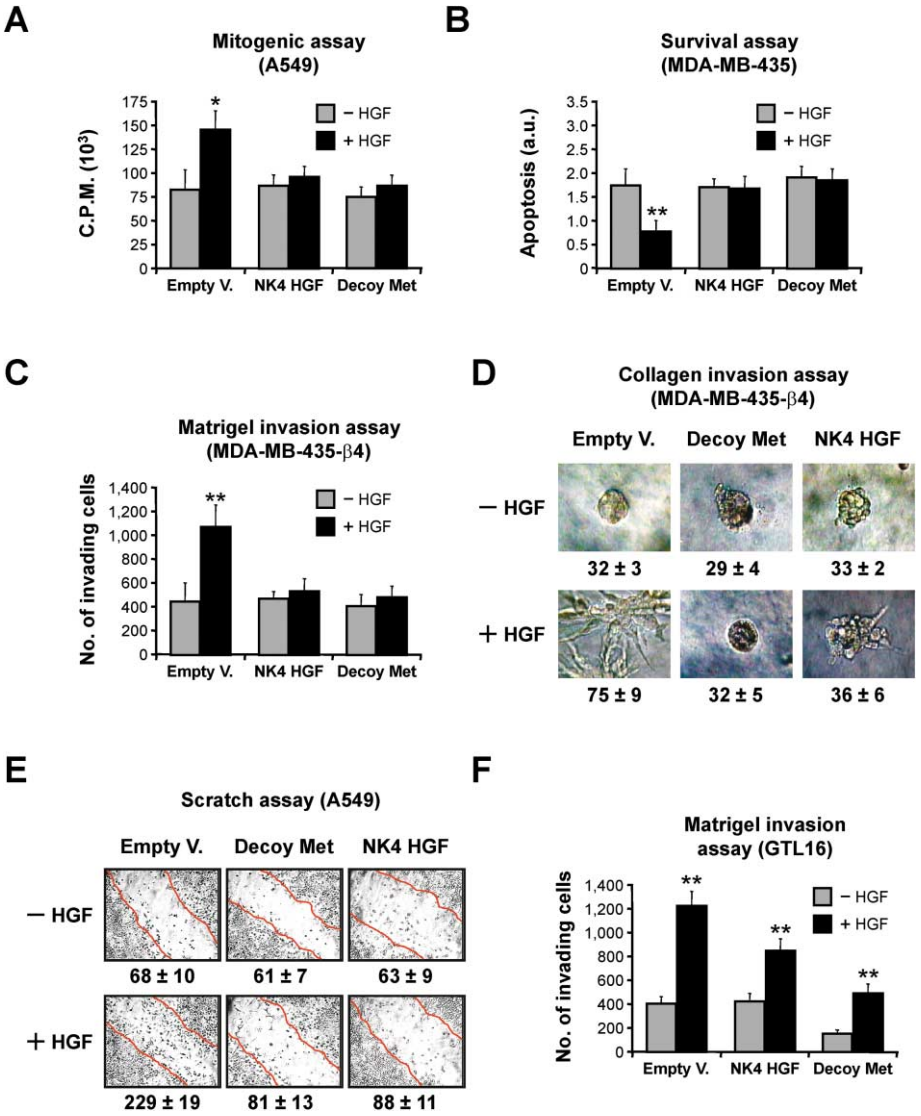

Figure 2. Decoy Met inhibits the pleiotropic activities of HGF on tumor cells

A: Mitogenic assay. Lentiviral vector-transduced tumor cells were stimulated with either recombinant HGF or no factor, and DNA synthesis was determined by [^{3}H]-thymidine incorporation. C.P.M., counts per minute; empty v., empty vector.

B: Survival assay. Tumor cells were preincubated with either recombinant HGF or no factor, and then cultured either in the presence or absence of staurosporine. Survival in the absence of staurosporine was not affected by NK4 HGF or decoy Met, and is not shown. Apoptosis is expressed in arbitrary units (a.u.).

C: Matrigel invasion assay. Tumor cells were analyzed for their ability to invade a Matrigel layer, either in the presence or absence of recombinant HGF.

D: Collagen invasion assay. Tumor cells were examined for their ability to form branched tubules in response to HGF stimulation. Values (mean ± SD) indicate the percentage of "sprouted" colonies over the total.

E: Scratch assay using a confluent cell monolayer. Cells were wounded and then incubated either in the presence or absence of recombinant HGF. Values (mean ± SD) indicate the number of cells that have migrated over the original margins (in red).

F: Matrigel invasion assay using tumor cells naturally overexpressing Met. The number of invading cells was determined as described above. Throughout the figures, statistical significance (experimental versus control value) is indicated by a single ($p < 0.05$) or double ($p < 0.01$) asterisk.

ated HUVEC migration and branching. In this regard, it should be noted that NK4 HGF has been found to inhibit angiogenesis independently of Met (Matsumoto and Nakamura, 2003), and to share remarkable structural homology with angiostatin (O'Reilly et al., 1994).

Decoy Met inhibits tumor growth and metastasis in mice

We next tested the biological effects of Met activation (by HGF) or inhibition (by NK4 HGF or decoy Met) in ex vivo tumorigenesis assays in mice. CD-1 *nu*$^{-/-}$ mice were injected subcutaneously with different tumor cell lines transduced in vitro with the various lentiviral vectors, and injected animals were monitored for tumor development. After 50 days, experimental tumors were extracted for analysis, and lungs were contrasted with India ink to highlight metastases. Following metastasis scoring, lungs were processed for histological analysis.

In experiments using MDA-MB-435 mammary carcinoma cells (Figure 4A), decoy Met dramatically reduced tumor weight compared to the control (5-fold, $p < 0.0001$). Also, NK4 HGF reduced tumor weight, although to a lesser extent (1.3-fold, $p = 0.0453$). Autopsy revealed that 40% of the animals in the control group developed pulmonary metastases, while all mice of the decoy Met group and 80% of the NK4 HGF group were metastasis-free. Symmetrically, mice injected with cells transduced with HGF lentiviral vector had a much higher tumor weight (1.9-fold, $p = 0.0023$) and developed more metastases (30.8-fold, $p = 0.0031$). Similar results were obtained with A549 lung carcinoma cells (Figure 4B), and with MDA-MB-435-β4 mammary carcinoma cells (Figure 4C), a more metastogenic variant of MDA-MB-435 (Trusolino et al., 2001).

Interestingly, histological analysis of lungs revealed remarkable biological differences between micrometastases found in the empty vector, NK4 HGF, and HGF groups (Supplemental Figure S5). In the control group, metastatic lesions were mostly small, parenchimatic, and sometimes infiltrated by lymphoid cells. In the NK4 HGF animals, micrometastases were poor in infiltrated cells, but appeared well delimited and highly apoptotic. In contrast, metastases in the HGF group appeared much larger, mostly embolic, and free of infiltrate. Furthermore, HGF

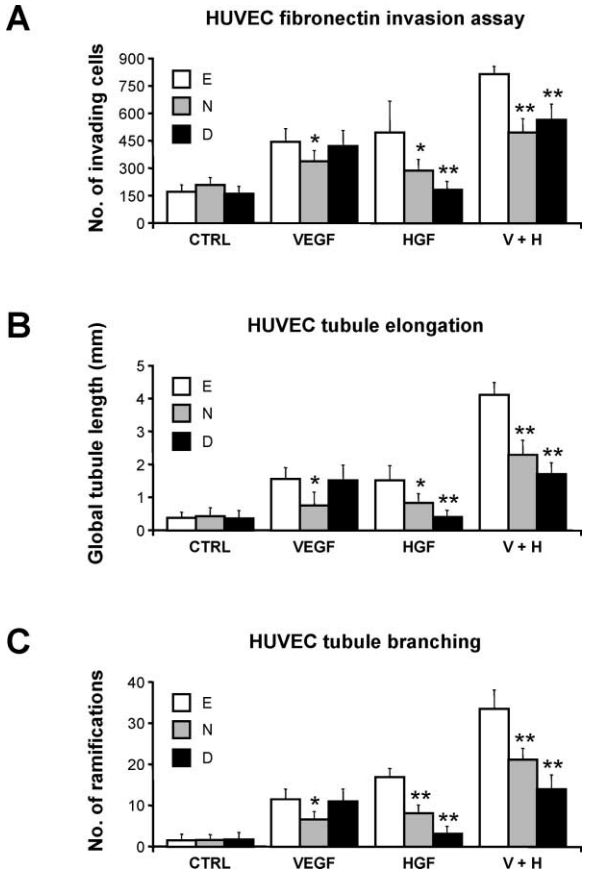

Figure 3. Decoy Met inhibits HGF-induced endothelial cell motility and morphogenesis

A: Fibronectin invasion assay. HUVECs were analyzed for their ability to invade a fibronectin layer in the presence of no factor, VEGF, HGF, or both HGF and VEGF. E, empty vector; N, NK4 HGF; D, decoy Met; CTRL, control; V + H, VEGF + HGF.

B: Branching morphogenesis assay. Preformed HUVEC spheroids were stimulated as in **A** and global tubule elongation was quantified by summing the length of all tubules in each colony analyzed.

C: Branched colony complexity from the experiment described in **B** is expressed by the number of branch points per colony (see Supplemental Figure S4 at http://www.cancercell.org/cgi/content/full/6/1/61/DC1 for representative images).

also promoted the formation of several "exploded" metastatic emboli invading the lung parenchima.

We wondered whether the absence of metastatic lesions in the decoy Met group was merely due to reduced tumor size. We therefore reperformed the same analysis using MDA-MB-435-β4 cells on a panel of randomized animals that were all bearing large tumors of the same volume (approximately 4,000 mm^3) at the time of sacrifice, regardless of the time required by the tumor to reach such volume (Figure 4D). In this analysis as well, no metastasis at all could be found in the decoy Met group, suggesting that decoy Met prevents tumor cell spreading directly and independently of its parallel effect on tumor growth.

Decoy Met blocks the growth and dissemination of established tumors

We next analyzed whether NK4 HGF or decoy Met could interfere with growth and invasion of an established tumor. To this end, we subcutaneously injected MDA-MB-435-β4 human mammary carcinoma cells into CD-1 *nu*$^{-/-}$ mice, and then monitored animals for tumor appearance. After two weeks, all mice bearing tumors of approximately 100 mm^3 were selected and randomly divided into four groups ($n = 6$). High-titer lentiviral vector preparations were injected intratumorally, and tumor volume was measured every third day. After 18 days, mice were subjected to autopsy.

While tumors injected with empty vector enlarged more than 6-fold during the course of the experiment, injection of NK4 HGF or decoy Met almost completely arrested the growth of the neoplastic mass (NK4 HGF, $p = 0.0001$; decoy Met, $p < 0.0001$; Figure 5A). Symmetrically, HGF accelerated tumor growth by 1.6 times compared to the control ($p = 0.0032$). Similar results emerge from analysis of tumor weight (Figure 5B). Immunofluorescence analysis of frozen tumor sections revealed widespread high expression of all the exogenous transgenes (Supplemental Figure S6). To determine the presence of metastatic lesions, we analyzed serial lung sections stained with hematoxylin and eosin. We found micrometastases in all experimental groups except decoy Met (Figure 5C).

We also determined the proliferative index (Ki67; Figure 5D) and apoptotic index (TUNEL; Figure 5E) of cells within the tumors. Remarkably, decoy Met reduced tumor cell proliferation by almost 2-fold ($p = 0.0001$) and increased apoptosis approximately 3.7 times ($p < 0.0001$). NK4 HGF also decreased tumor cell mitotic index (2.1-fold, $p < 0.0001$) and augmented apoptotic cell death (2.3-fold, $p < 0.0001$). HGF achieved the opposite effect on both proliferation (1.2-fold higher, $p = 0.0015$) and apoptosis (3-fold lower, $p < 0.0001$).

Decoy Met inhibits angiogenesis and impairs tumor vessel arborization

To determine vessel distribution within the transduced neoplastic lesions described above, we stained tumor sections with antibodies against the CD31 endothelial marker, and analyzed them by fluorescence microscopy. Three distinct parameters were determined (Figure 5F): vessel density, total vessel area, and mean lumen area. Strikingly, this analysis revealed that HGF increased vessel density by 1.7 times ($p = 0.0134$), but at the same time decreased the size of lumina by approximately the same order ($p = 0.0046$), thus not significantly changing total vessel area. In other words, HGF caused tumor vessels to be higher in number, but smaller in diameter, thus resulting in greater arborization. Conversely, decoy Met decreased vessel density by approximately 11 times ($p = 0.0178$), but reduced total vessel area only 3.2-fold ($p = 0.0224$), thus resulting in dramatically higher lumen caliber (approximately 3.5-fold, $p = 0.0192$; see Figure 5G for representative images). Interestingly, and consistent with a different mechanism of angiogenesis inhibition, NK4 HGF reduced both vessel density (1.4-fold, $p = 0.0017$) and mean lumen area (1.4-fold, $p = 0.0016$), thus resulting in fewer *and* smaller vessels within the tumor.

Concomitant action on tumor and endothelial cells by decoy Met results in optimal inhibition of tumor growth

To determine whether the sole inhibition of vessel arborization by decoy Met could have biological consequences on tumor growth, we performed ex vivo tumorigenesis assays using the TOV-112D human ovarian carcinoma cell line, which lacks Met

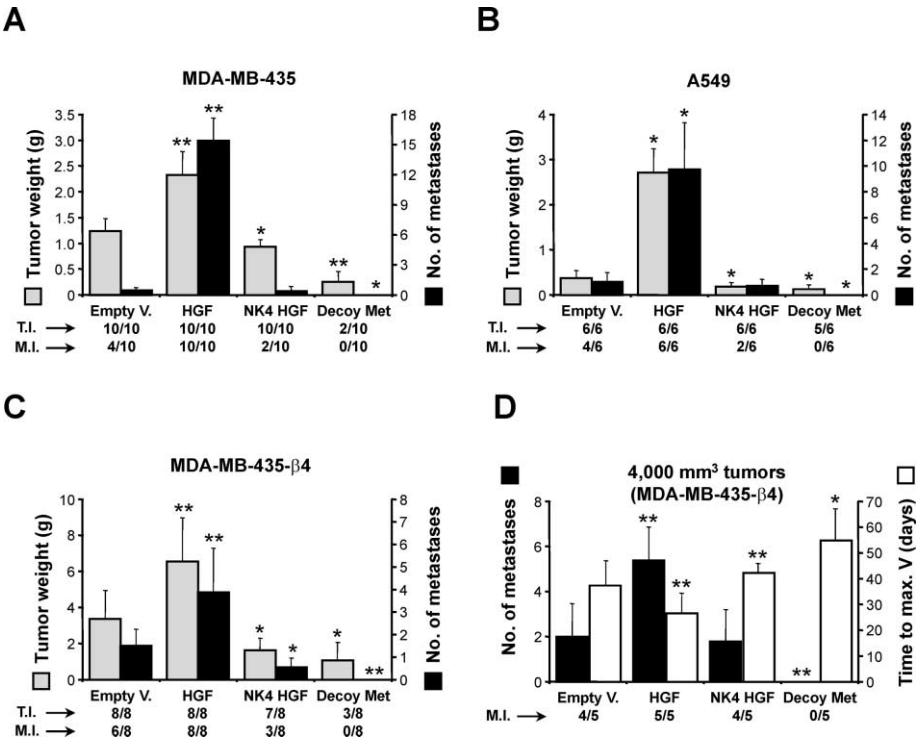

Figure 4. Ex vivo expression of decoy Met inhibits tumor growth and metastasis in mice

A–C: Cells were transduced with the indicated lentiviral vector and then injected subcutaneously into nude mice. After eight weeks, tumor weight (left Y axis, gray) and pulmonary metastases (right Y axis, black) were determined. Empty v., empty vector. T.I., tumor incidence; M.I., metastasis incidence. See also Supplemental Figure S5 at http://www.cancercell.org/cgi/content/full/6/1/61/DC1 for metastasis histology.

D: Metastasis analysis on mice bearing MDA-MB-435-β4 tumors measuring 4,000 mm^3 at the time of lung extraction. Left Y axis, number of metastases (black); right Y axis, time required by tumors to reach a 4,000 mm^3 volume (white).

expression as determined by both Western blot and RT-PCR analysis (Figure 6A). We transduced TOV-112D cells with the full panel of lentiviral vectors, and then injected them subcutaneously into nude mice. Tumor growth was monitored as described above for 20 days, and then tumors were extracted for analysis.

We first determined whether mouse vessels within the tumor were expressing the Met receptor. Confocal immunofluorescence analysis revealed complete signal overlapping between CD31 and mouse Met staining (Figure 6B). By RT-PCR analysis, we also determined that cancer cells in all tumors had not acquired *met* expression during the course of the experiment (Supplemental Figure S7A).

Next, we determined vessel distribution as performed for MDA-MB-435-β4 tumors using anti-CD31 antibodies (Figure 6C). We observed that: (1) decoy Met decreased vessel density (5-fold, $p < 0.0001$), increased mean lumen area (2.1-fold, $p = 0.0033$), and reduced total vessel area (2.4-fold, $p = 0.0002$); (2) NK4 HGF decreased vessel density (2-fold, $p = 0.0003$), mean lumen area (1.3-fold, $p = 0.0311$), and total vessel area (2.7-fold, $p < 0.0001$); and (3) HGF increased vessel density (1.7-fold, $p = 0.0006$) and decreased mean lumen area (1.5-fold, $p = 0.0065$), but did not significantly augment total vessel area (1.1-fold, $p = 0.0769$).

This remarkable impairment of vessel morphology by decoy Met resulted in a net 20% decrease in tumor incidence and in a significant delay of tumor formation (>2.4-fold, $p < 0.0029$; Figure 6D). Similarly, inhibition of vessel formation by NK4 HGF also decreased tumor incidence by 20%, and significantly delayed tumor formation (>2.2-fold, $p < 0.0259$). Consistent with these observations, promotion of vessel arborization by HGF significantly accelerated tumor formation (2-fold, $p = 0.0352$). However, once they appeared, all tumors of the decoy Met and NK4 HGF groups seemed to have recovered from their initial reserve, and grew at a high rate. By the end of the experiment, no statistically significant difference in tumor volume or weight could be measured between the decoy Met or NK4 HGF group and the empty vector group. In contrast, HGF dramatically increased both tumor volume (5.3 times, $p = 0.0422$) and weight (4.9 times, $p = 0.0378$). Western blot analysis on tumor lysates ruled out the possibility that cancer cells had lost decoy Met or NK4 HGF expression during the course of the experiment (Supplemental Figure S7B).

This phenomenon can be explained if we hypothesize that inhibition of angiogenesis by decoy Met or NK4 HGF applies a selective pressure on tumor cells. During an early "crisis" phase, cancer cells that have a reduced vascular demand (Rak et al., 2002) are selected for, and subsequently take over the remaining tumor cell population. In contrast, HGF provides an advantage to tumor cells, because it promotes angiogenesis. All in all, we can conclude that: (1) HGF sustains the growth and expansion of a tumor, even when tumor cells do not express the HGF receptor, because it promotes host vessel arborization; and (2) the sole impairment of angiogenesis by decoy Met or NK4 HGF—without a concomitant action on tumor cells—is insufficient to determine a persistent inhibition of tumor growth, at least in the biologic system analyzed.

Systemic inhibition of HGF/Met prevents tumor growth and metastasis without substantially affecting housekeeping physiological functions

In our next approach, we compared the effects of systemic HGF/Met inhibition on tumor versus normal tissues. Systemic administration of lentiviral vectors is a powerful approach to obtain sustained plasmatic levels of a secreted factor (Follenzi et al., 2002). To this end, we injected i.v. a panel of CD-1 $nu^{-/-}$

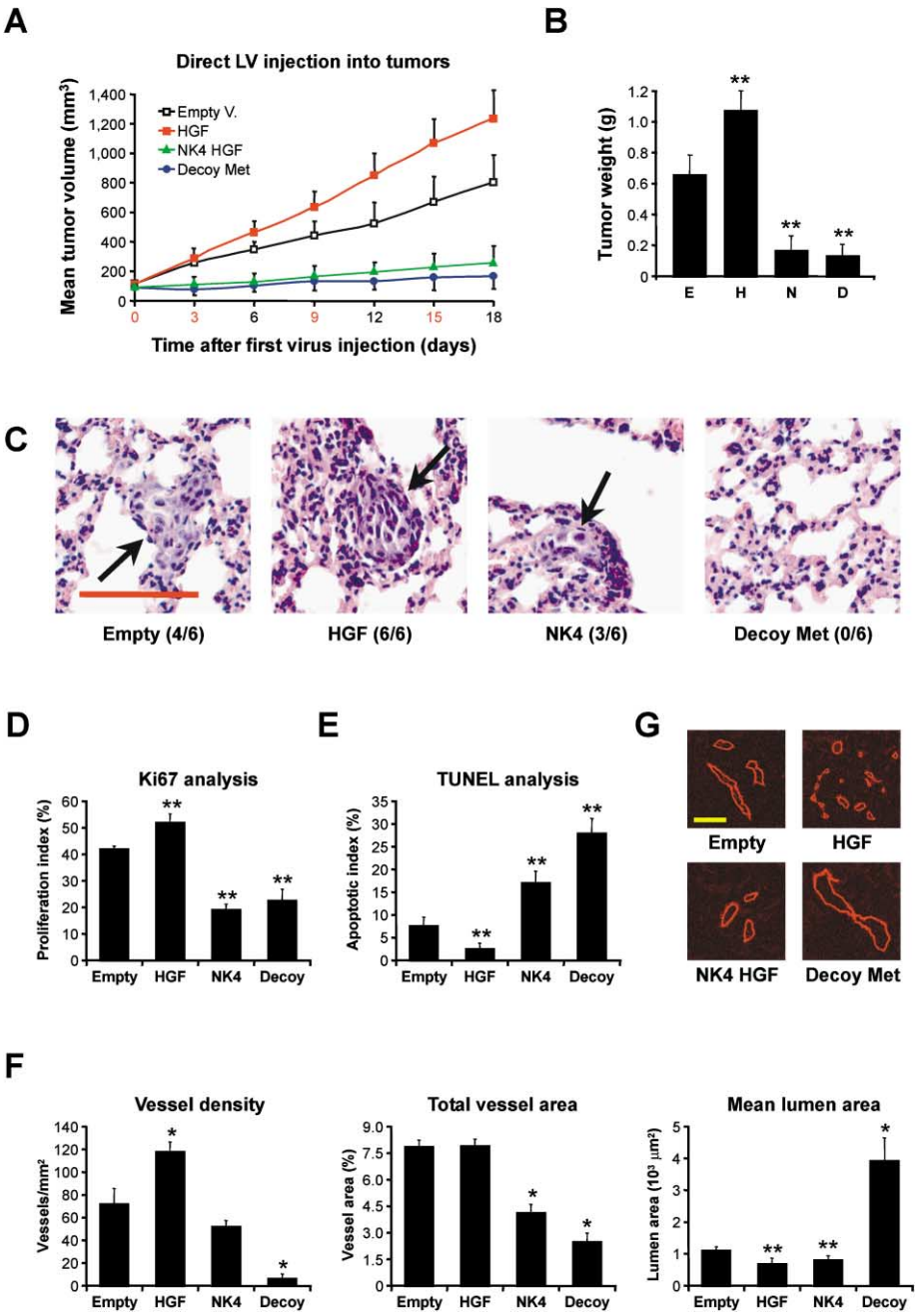

Figure 5. Decoy Met inhibits the growth, vascularization, and dissemination of established tumors

A: Tumor burden analysis. Tumors of the same size (approximately 100 mm^3 at day 0) were repeatedly injected with the indicated lentiviral vectors, and tumor volume was measured every 3 days. The numbers in red correspond to the days of lentivirus injection. LV, lentiviral vector.

B: Tumor weight analysis of the experiment described in **A**. E, empty vector; H, HGF; N, NK4 HGF; D, decoy Met.

C: Histological analysis of pulmonary metastases on lung sections stained with hematoxylin and eosin. A representative microscopic field for each group is shown. Arrows indicate the position of the metastatic lesion. Metastasis incidence is shown in parentheses. The red bar is a 100 μm marker.

D: Tumor proliferation index analysis (% Ki67-positive cells).

E: Tumor apoptotic index analysis (% TUNEL-positive cells).

F: Tumor vessel analysis: vessel density, number of vessels per mm^2; total vessel area, percentage of the tumor section occupied by vascular tissue; mean lumen area, average section of a vessel in μm^2.

G: Representative images of the analysis described in **F**. The yellow bar is a 100 μm marker.

mice with highly concentrated lentiviral vector preparations, and obtained three distinct sets of animals expressing stable (Supplemental Figure S8 at http://www.cancercell.org/cgi/content/full/6/1/61/DC1) picomolar plasma concentrations of HGF (40–100 pM), NK4 HGF (24–41 pM), or decoy Met (11–50 pM), plus one set of control animals (empty vector). Considering that endogenous HGF is found in normal mice at about 5–6 pM (Xue et al., 2002), the observed factor concentrations are certainly significant from a biological viewpoint.

Between three and five weeks after injection, mice were subjected to a series of tests aimed at determining whether sustained expression of exogenous HGF or HGF antagonists was perturbing their major housekeeping physiological functions. We measured various clinical and biological parameters, including ability to repair a cutaneous wound, complete hemochrome and white blood cell formula, hemoglobin concentration, coagulation properties, liver and kidney function enzymes, and other markers (Table 1 and Supplemental Table S1). This analysis revealed that most physiological parameters were left substantially unaffected, with a few remarkable exceptions in the NK4 HGF group. In fact, NK4 HGF increased both red (1.2-fold, $p < 0.0001$) and white (1.1-fold, $p = 0.0279$) blood cell numbers, while it decreased platelet concentration (1.2-fold, $p = 0.0278$). Microscopy analysis revealed that this increment in white blood cells was contributed mainly by granulocytes (not shown). NK4 HGF also increased hemoglobin concentration

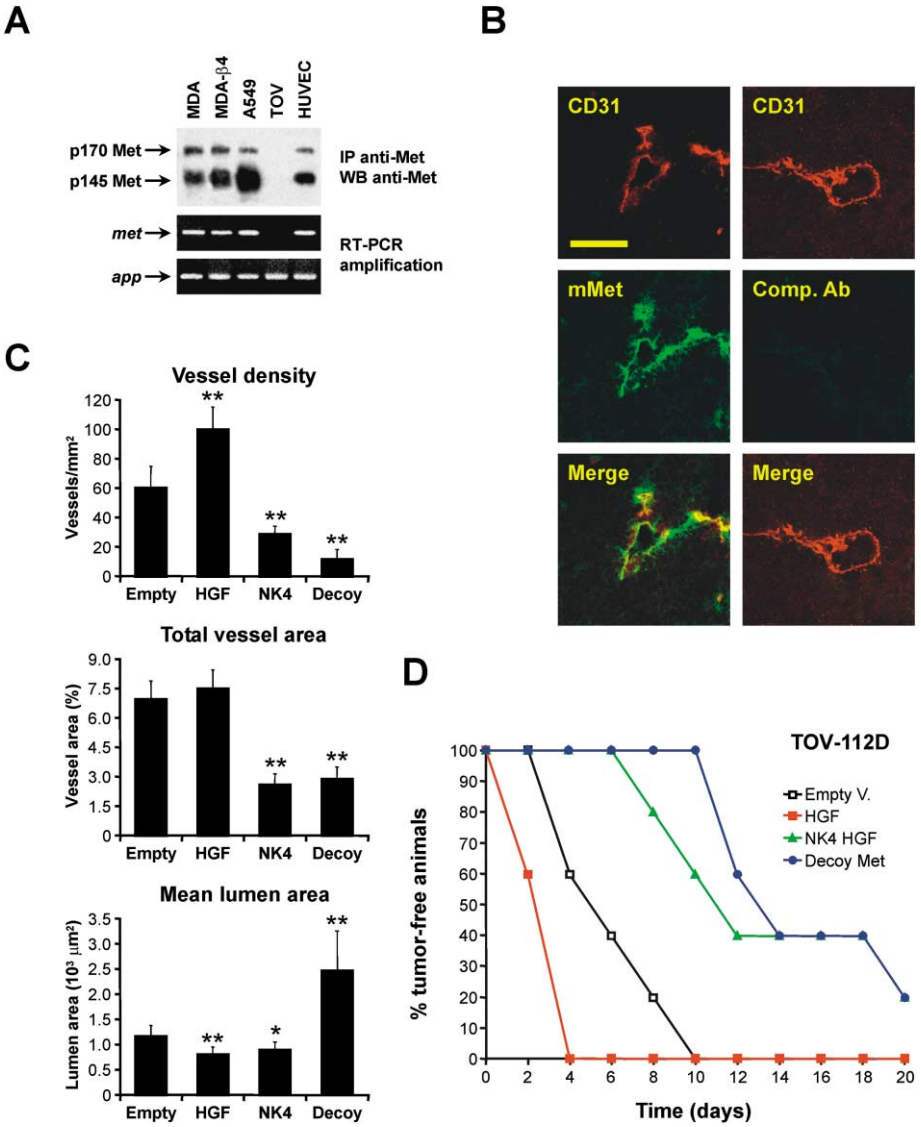

Figure 6. Decoy Met delays the growth of tumor cells lacking the HGF receptor by impairing host vessel arborization

A: Analysis of Met protein and *met* mRNA expression in human tumor cell lines and primary endothelial cells. WB, Western blot; IP, immunoprecipitation; RT-PCR, reverse transcriptase polymerase chain reaction; *app*, human housekeeping gene encoding β-amyloid precursor protein (see also Supplemental Figure S5 at http://www.cancercell.org/cgi/content/full/6/1/61/DC1).

B: Confocal immunofluorescence analysis of CD31 and mouse Met expression on TOV-112D tumors. Frozen sections were costained with anti-mouse CD31 antibodies (in red) and anti-mouse Met antibodies (plain or epitope-competed; in green). The yellow bar is a 100 μm marker.

C: Tumor vessel analysis of TOV-112D tumor sections. CD31 staining was analyzed as described for Figure 5F.

D: Tumorigenesis assay using lentiviral vector-transduced TOV-112D cells. Mice were injected with lentiviral vector-injected cells and then monitored every two days for tumor appearance. Empty v., empty vector.

(1.1-fold, $p = 0.0004$; Table 1). Since these changes were not observed in the decoy Met group, nor was a symmetric effect elicited by HGF, hemochrome alterations induced by NK4 HGF are conceivably a result of Met-independent mechanisms. No other parameter analyzed was significantly changed compared to the control, either in the NK4 group or in the other two.

Approximately five weeks after lentiviral vector injection, transduced mice were injected subcutaneously with MDA-MB-435-β4 cells, and tumor formation was monitored as already described. In striking contrast with the results obtained by examining housekeeping physiological functions, analysis of tumor formation (Table 1 and Supplemental Figure S9A) revealed that mice expressing picomolar concentrations of decoy Met were less permissive to xenograft implantation, as they displayed longer latency and reduced penetrance. Also, tumor growth was substantially inhibited, as measured by volume increase over time. Conversely, consistent with the previous in vivo data, mice expressing exogenous HGF displayed accelerated tumor formation and a higher neoplastic growth rate. In the NK4 group, tumor latency was slightly increased, and tumors grew at a lower rate. However, tumor incidence was not reduced, suggesting that the plasmatic concentrations of NK4 HGF reached in these mice—in contrast with those obtained locally by direct tumor injection—may be too low to achieve a significant tumor inhibition.

Autopsy (see Table 1) was performed 46 days after tumor cell injection. While all mice developed visible pulmonary lesions in the control, NK4 HGF, and HGF groups, no metastasis could be detected in the decoy Met mice except for one animal. NK4 HGF did not reduce metastasis incidence, but only metastasis number. Metastases in the HGF group were not only higher in number, but also larger and more invasive compared to the empty vector group (see Supplemental Figure S9B for representative images). Histological analysis of liver and kidney sections did not reveal any obvious sign of pathology (not shown). In contrast, consistent with a role of HGF in hemopoiesis (Galimi et al., 1994), bone marrow cell apoptotic index was found to be increased by NK4 HGF and decoy Met, and decreased by HGF (Table 1).

Table 1. Sustained picomolar plasma concentrations of decoy Met inhibit tumor growth and prevent metastasis without significantly affecting mouse physiology

	Exp. group and mouse no.	FPC (pM)	Tumor latency (days)	Tumor weight (g)	Number of pulmonary metastases	WH time (days)	Bone marrow a.i. (%)	RBC (10^6/ml)	WBC (10^3/ml)	PLT (10^3/ml)	HGB (mg/dl)
Empty vector	#1	0.0	8.0	5.36	4.0	9.0	5.58	7.59	2.20	780.0	16.1
	#2	0.0	10.0	5.40	17.0	11.0	5.06	7.60	2.37	650.0	17.0
	#3	0.0	8.0	3.72	9.0	11.0	4.76	7.80	2.44	590.0	16.8
	#4	0.0	14.0	1.68	8.0	9.0	5.56	7.10	2.71	620.0	17.0
	#5	0.0	10.0	8.67	16.0	9.0	5.41	8.00	2.30	610.0	17.2
	Average	**0.0**	**10.0**	**4.97**	**10.8**	**9.8**	**5.27**	**7.62**	**2.40**	**650.0**	**16.8**
	SD	0.0	2.4	2.57	5.5	1.1	0.35	0.33	0.19	75.8	0.43
	p	N/A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
HGF	#1	100.2	6.0	9.98	23.0	9.0	4.05	9.10	2.47	700.0	16.8
	#2	46.4	8.0	9.34	25.0	11.0	3.53	7.10	2.03	730.0	16.1
	#3	40.8	8.0	6.98	16.0	11.0	3.95	9.10	2.71	640.0	17.2
	#4	80.8	8.0	7.89	32.0	9.0	4.30	8.20	2.37	620.0	16.7
	#5	70.2	6.0	7.32	18.0	9.0	3.81	8.10	2.37	650.0	16.8
	Average	**67.7**	**7.2**	**8.30**	**22.8**	**9.8**	**3.93**	**8.32**	**2.39**	**668.0**	**16.7**
	SD	24.6	1.1	1.30	6.30	1.1	0.29	0.83	0.24	45.5	0.4
	p	N/A	*0.048*	*0.032*	*0.013*	1.000	*<0.001*	0.118	0.922	0.661	0.711
NK4 HGF	#1	26.9	16.0	2.20	1.0	9.0	6.21	8.90	2.64	810.0	18.6
	#2	36.8	14.0	2.59	5.0	11.0	5.88	9.10	2.71	720.0	18.2
	#3	27.7	10.0	1.98	7.0	9.0	6.86	8.90	3.05	710.0	17.9
	#4	24.2	18.0	3.26	13.0	11.0	6.25	8.90	2.54	830.0	17.9
	#5	41.0	8.0	3.20	2.0	9.0	6.15	9.30	2.71	740.0	18.4
	Average	**31.3**	**13.2**	**2.65**	**5.6**	**9.8**	**6.27**	**9.02**	**2.73**	**762.0**	**18.2**
	SD	7.2	4.1	0.58	4.8	1.1	0.36	0.18	0.19	54.5	0.3
	p	N/A	0.176	0.084	0.151	1.000	*0.002*	*<0.001*	*0.028*	*0.028*	*<0.001*
Decoy Met	#1	20.5	20.0	0.89	0.0	11.0	7.89	8.40	2.30	730.0	17.0
	#2	21.9	12.0	1.71	2.0	9.0	7.97	9.40	2.10	670.0	18.8
	#3	13.2	>46.0	0.02	0.0	11.0	8.04	7.30	2.06	710.0	16.2
	#4	50.5	20.0	0.52	0.0	11.0	7.14	7.50	2.74	690.0	17.0
	#5	11.5	22.0	0.35	0.0	11.0	9.03	8.90	2.20	760.0	16.2
	Average	**23.5**	**>24.0**	**0.70**	**0.4**	**10.6**	**8.01**	**8.30**	**2.28**	**712.0**	**17.0**
	SD	15.7	12.8	0.65	0.9	0.9	0.67	0.90	0.27	34.9	1.1
	p	N/A	*<0.044*	*0.007*	*0.003*	0.242	*<0.001*	0.150	0.431	0.135	0.679

CD-1 *nu*$^{-/-}$ mice were injected i.v. with equal amounts of the indicated lentiviral vector and then assayed for the presence of the appropriate factor in plasma (FPC, factor plasma concentration; see also Supplemental Figure S8 at http://www.cancercell.org/cgi/content/full/6/1/61/DC1). For each experimental group, 5 mice expressing sustained picomolar levels of factor were selected, and then injected subcutaneously with tumor cells. The formation of experimental tumors and of spontaneous metastases was analyzed as described in text. The same mice were subjected to a series of tests aimed at determining several biological and clinical parameters (WH, wound healing; a.i., apoptotic index; RBC, red blood cells; WBC, white blood cells; PLT, platelets; HGB, hemoglobin). Additional clinical parameters are analyzed in Supplemental Table S1.
Statistical significance (p value) was calculated by a two-tail homoscedastic Student's t test. Significant values ($p < 0.05$) are shown in italic. As revealed by power calculation, this analysis detects variations corresponding to 2-fold the standard deviation (SD) with a power of 80%. N/A, not applicable.

Decoy Met synergizes with radiotherapy in inducing tumor regression

HGF/Met signaling has been implicated in chemo- and radioresistance of tumors (Fan et al., 1998). We therefore tested whether decoy Met could sensitize tumor cells to radiotherapy. We irradiated lentiviral vector-transduced MDA-MB-435-β4 cells in vitro, and determined their survival in the presence or absence of recombinant HGF (Figure 7A). As expected, decoy Met efficiently abrogated the ability of HGF to protect tumor cells against the toxic effects of radiation. NK4 HGF was also effective in this system, although to a lesser extent.

Next, we injected wild-type MDA-MB-435-β4 cells into CD-1 *nu*$^{-/-}$ mice, and monitored animals for tumor appearance. Mice bearing tumors of approximately 100 mm^3 were selected and randomly divided into four groups (n = 6) as described above. Each group was injected intratumorally with the appropriate lentiviral vector on day 0 and 3, and mice were given a subcurative irradiation dose (15 Gy) on day 6. After irradiation, tumor volume was monitored for additional 18 days. Tumors transduced with empty vector partially regressed shortly after irradiation, but eventually relapsed and started to grow again within a week (Figure 7B). Tumors transduced with HGF displayed little if any radiosensitivity. In contrast, tumors transduced with decoy Met or NK4 HGF constantly regressed following irradiation and did not relapse. At the end of the experiment (day 24 after virus injection), tumors transduced with empty vector or HGF had increased their size by 2.2- and 6.7-fold, respectively, while tumors transduced with decoy Met or NK4 HGF had regressed by 4.7- and 2.8-fold, respectively. Thus, HGF protects tumor cells against radiation; conversely, inhibition of the HGF/Met pathway by decoy Met or NK4 HGF synergizes with radiotherapy in inducing tumor regression.

Discussion

In this study, we utilized a lentiviral vector-based gene transfer approach to investigate the role of HGF in tumor progression,

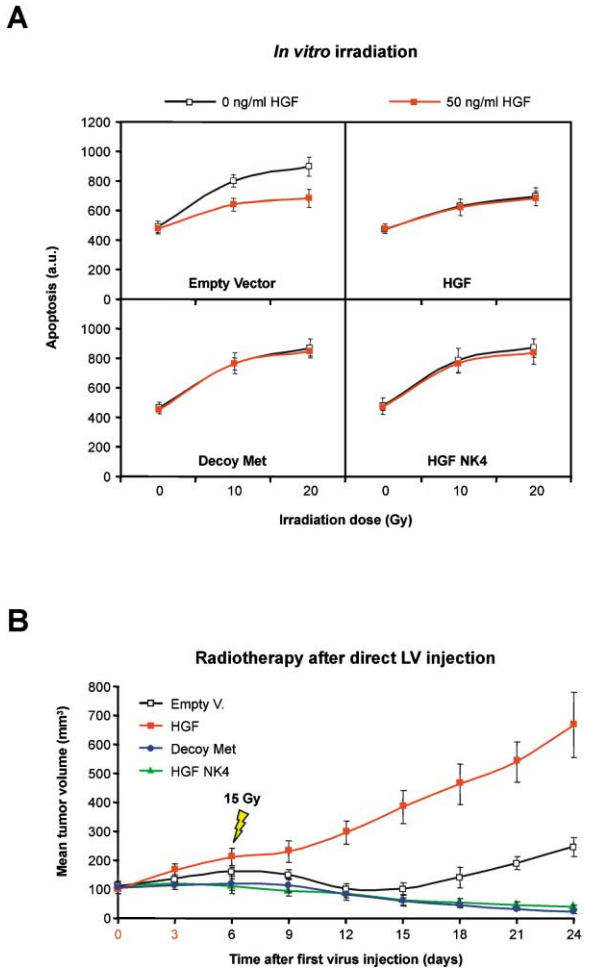

Figure 7. Decoy Met sensitizes tumor cells to radiotherapy

A: Lentiviral vector-transduced MDA-MB-435-β4 cells were irradiated (0, 10, 20 Gy) in the presence or absence of recombinant HGF, and apoptosis was determined after 48 hr. Apoptosis is expressed in arbitrary units (a.u.). **B:** Tumors of the same size (approximately 100 mm^3) were injected with the indicated lentiviral vectors at days 0 and 3, and then irradiated at day 6 (15 Gy). Tumor volume was measured every 3 days. The numbers in red correspond to the days of lentivirus injection. LV, lentiviral vector.

and to explore the therapeutic potential of HGF/Met inhibitors in antineoplastic therapy.

As mentioned in the Introduction, multiple approaches have been previously attempted to inhibit HGF or Met in experimental systems. Among all the explored routes, the use of NK4 HGF to displace HGF from Met is certainly the most thoroughly characterized and perhaps the most promising. The in vitro and preclinical data presented in our study suggest, however, that decoy Met has several biochemical and biological advantages over NK4 HGF. First, decoy Met binds to HGF with approximately one log higher affinity than NK4 HGF binds to Met. Second, decoy Met can also inhibit Met activation induced by ligand-independent mechanisms. Third, decoy Met is significantly more effective than NK4 HGF in preventing metastasis, and has less pronounced systemic side effects.

In addition, decoy Met may also present some advantages over other HGF/Met inhibitors. For example, neutralizing monoclonal antibodies against HGF showed significant antitumoral activity in experimental animals (Cao et al., 2001). However, tumor inhibition was achieved only with a combination of at least three different monoclonal antibodies, each for a distinct epitope of HGF. Since each monoclonal antibody may have its own specificity, pharmacodynamics, biodistribution, side effects, and dose/effect curve, the clinical application of this approach may encounter some complications. As a further concern, neutralizing antibodies against HGF cannot prevent Met activation induced by receptor overexpression, a very common event in human cancer.

A different approach achieved downregulation of HGF and/or Met in various experimental systems via U1snRNA/ribozymes (Jiang et al., 2001). This technique makes use of a triple combination of a U1snRNA, a hammerhead ribozyme, and an antisense mRNA sequence to downregulate the messenger RNA of a given gene. While this approach may be a valuable tool in target validation studies that employ in vitro engineered cell lines, it cannot have realistic clinical applications as of now, since it is difficult to imagine how to achieve an efficient delivery of the specific ribozyme to every single tumor cell in a patient. Decoy Met, in contrast, is a secreted protein, which makes it possible to achieve a paracrine and/or endocrine effect on all the cellular components of the tumor by gene transfer to only a small fraction of cells. Similar arguments apply to the use of transmembrane, dominant-negative forms of Met (Firon et al., 2000; Furge et al., 2001).

Recently, small molecule inhibitors against the Met kinase have been developed (Sattler et al., 2003; Christensen et al., 2003; Wang et al., 2003). These compounds prevent ATP from binding to the ATP binding site of Met. This is an interesting pharmacological approach that has achieved successful results for other tyrosine kinases. However, small molecules inhibitors also have their own limitations. First, no ATP analog will ever be absolutely specific for a given tyrosine kinase, and thus, toxicity is a big concern. A ligand-receptor interaction is, in contrast, extremely specific. Second, the biological activity of small molecule inhibitors is dependent on a series of pharmacological and pharmacodynamical parameters, such as biodistribution, bioavailability, membrane permeability, clearance, and metabolism, that have to be tested case by case in both preclinical and clinical settings. In the case of the mentioned molecules, no information on these parameters is yet available. Furthermore, none of the quoted studies analyzed metastasis, angiogenesis, or the effect of these anti-Met compounds on normal physiologic functions.

Our preclinical data indicate that HGF levels greatly influence the likelihood of developing metastases. Since HGF is ubiquitous in tissues, and its plasma levels fluctuate in relation to particular physiological or pathological conditions, our findings have important implications in cancer therapy. For example, oncological patients who may potentially bear residual tumor cells—following primary tumor resection and/or chemotherapy—may be treated with anti-HGF agents to prevent the growth of such cells into manifest metastatic lesions. Alternatively, premalignant lesions for which surgical removal may not be appropriate could be held in check by HGF antagonists, in order to prevent their malignant conversion. With regard to these possibilities, our data indeed demonstrate that decoy Met inhibits metastatic spread (1) independently of tumor size, (2) from

preexisting neoplastic lesions, and (3) as a preventive agent in circulating picomolar concentrations.

While anti-HGF/Met drugs may be very efficient in preventing tumor progression and metastasis, inhibition of the HGF/Met pathway per se may not be sufficient to cause tumor regression. However, our results demonstrate that decoy Met or NK4 HGF significantly sensitize tumor cells to irradiation, and synergize with radiotherapy in promoting tumor regression. This finding significantly broadens the therapeutic potential of HGF/Met antagonists. In fact, anti-Met drugs may be used in combination with conventional chemo- or radiotherapy regimes to increase tumor sensitivity, thereby amplifying the therapeutic effect.

Our in vivo experiments show that systemic inhibition of HGF/Met in adult animals does not significantly affect housekeeping physiologic functions, but dramatically impairs tumor growth and prevents the formation of metastases. Although the substantial absence of side effects in our study does not rule out that more subtle differences could be detected by employing a more powerful analysis, or that higher concentrations of inhibitors could elicit more significant changes, our data demonstrate that tumors are more sensitive to anti-Met drugs than normal tissues. The differential effect of HGF/Met inhibition on tumor versus normal tissues could easily be explained by—and is reasonably a proof of—the hypothesis that tumors are nothing but "miniorgans" attempting to organize themselves and expand (Bissell and Radisky, 2001). With regard to this concept, it is noteworthy to observe that *hgf* or *met* knockout mice die in utero due to liver failure and placental defects, resulting from impaired trophoblast invasion of uterine matrix and abnormal vascularization of corial villi (Schmidt et al., 1995; Uehara et al., 1995; Bladt et al., 1995). Thus, HGF-mediated invasive cues are essential during embryonic organ formation, but may be dispensable under normal circumstances in the adult, unless a tumor "smartly" resumes a discontinued genetic program to establish itself and its progeny into the host organism.

Experimental procedures

Ex vivo tumorigenesis assays

Lentiviral vector-transduced tumor cells were injected subcutaneously into the right posterior flank of six-week-old immunodeficient $nu^{-/-}$ female mice on Swiss CD1 background (Charles River Laboratories, Calco, Lecco, Italy). The number of cells injected per mouse varied upon cell line (MDA-MB-435 cells, 2×10^6; MDA-MB-435-β4 cells, 1×10^6; A549 cells, 4×10^6; TOV-112D cells, 6×10^6). Tumor appearance was evaluated every 2 days using a caliper. Tumor volume was calculated using the formula $V = 4/3\pi \times (d/2)^2 \times D/2$, where d is the minor tumor axis and D is the major tumor axis. A mass of 15 mm^3—corresponding approximately to the initial volume occupied by injected cells—was chosen as threshold for tumor positivity. At the end of the observation period, tumors were weighed, and lung nodules were contrasted with black India ink to facilitate scoring. Superficial pulmonary metastases were counted on dissected lung lobes under a stereoscopic microscope. Histological analysis was performed on paraffin-embedded sections stained with hematoxylin and eosin. All animal procedures were approved by the Ethical Commission of the University of Torino, Italy, and by the Italian Ministry of Health.

Direct injection of lentiviral vectors into preformed tumors

MDA-MB-435-β cells were injected subcutaneously (2×10^6 cells/mouse) into $nu^{-/-}$ mice as described above. After 2 weeks, mice bearing a tumor of approximately 100 mm^3 were selected, and randomly divided into 4 groups of 6 mice each. At this time (day 0) and on days 3, 9, and 15, high-titer lentiviral vector preparation (1 μg p24/mouse) was administrated intratumorally using a Hamilton syringe (Hamilton, Reno, Nevada). Tumor volume was measured every 3 days. At day 18, mice were killed by CO_2 inhalation; tumors were excised and weighed. Half of the tumor was embedded in Tissue-Tek OCT compound (Sakura Finetek, Torrance, California), and immediately frozen in liquid nitrogen. The second half of the tumor was embedded in paraffin. Tumor sections were used for tissue analysis. Lungs were embedded in paraffin, serially sectioned, and stained with hematoxylin and eosin for histological analysis.

Systemic delivery of lentiviral vectors

Six-week-old nude female mice (6 mice/group) were injected through the tail vein with the appropriate concentrated lentiviral vector (20 μg p24 HIV equivalents/mouse). After three weeks, plasma concentrations of recombinant proteins were estimated by ELISA (see Supplemental Experimental Procedures at http://www.cancercell.org/cgi/content/full/6/1/61/DC1). Mice expressing the lowest factor levels were excluded (1 mouse/group). Blood samples were periodically obtained from these mice for up to 12 weeks. Plasma factor concentrations as well as a series of clinical parameters were determined. Approximately 5 weeks after lentiviral vector injection, MDA-MB-435-β4 cells were injected subcutaneously as described above (2×10^6 cells/mouse), and tumor burden was measured every 2 days. After an observation time of 46 days (approximately 12 weeks after lentivirus injection), mice were subjected to autopsy. Metastases were scored as described above. Tumors were excised, weighed, embedded in Tissue-Tek OCT compound, and immediately frozen in liquid nitrogen. Bone marrow was harvested from femora and suspended in PBS plus 2% FBS for cytological analysis. Livers and kidneys were embedded in OCT (to assess transduction efficiency) and in paraffin (for histological analysis).

Supplemental data

For all experimental procedures not listed here above, please refer to the Supplemental Experimental Procedures at http://www.cancercell.org/cgi/content/full/6/1/61/DC1.

Acknowledgments

We are grateful to Elisa Vigna, Antonia Follenzi, Michele De Palma, and Mary Venneri for assistance with lentiviral vectors, Selma Pennacchietti, Livio Trusolino, Paolo Conrotto, Andrea Rasola, and Guido Serini for precious reagents and advice, Pietro Gabriele, Barbara Baiotto, and Victor Macias for cell and mouse irradiation, Mauro Risio and Giovanni De Rosa for histological consultation, and Ada Arnesano and Enrica Charbonnier for skilled technical assistance. This work has been supported in part by the Italian Association for Cancer Research (AIRC), the Compagnia San Paolo di Torino Foundation, and the Italian Ministry of University and Research (MIUR COFIN # 2001068458).

Received: December 11, 2003
Revised: April 19, 2004
Accepted: April 20, 2004
Published: July 19, 2004

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