Suppression of Ras-mediated tumorigenicity and metastasis through inhibition of the Met receptor tyrosine kinase

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Mutations in the Ras family of GTP binding proteins represent one of the most frequently observed genetic alterations in human cancers. We and others have recently demonstrated that expression of Met, the tyrosine kinase receptor for hepatocyte growth factor/scatter factor (HGF/SF), is significantly up-regulated in Ras-transformed cells. Because HGF/SF-Met signaling is proposed to play a prominent role in tumor development and progression, we assessed the possible requirement for Met during Ras-mediated tumor growth and metastasis. To disrupt endogenous Met signaling, we constructed dominant-negative mutants of both human and murine Met and showed that these can inhibit HGF/SF-mediated Met signaling and cell invasion of *ras*-transformed cells *in vitro*. Moreover, ectopic expression of dominant-negative Met mutants reduced the s.c. tumor growth of *ras*-transformed cells and dramatically suppressed their ability to form lung metastases *in vivo*. Our data demonstrate that Met plays a prominent role during Ras-mediated tumor growth and metastasis, and further suggest that agents that inhibit HGF/SF-Met signaling may represent an important therapeutic avenue for the treatment of a variety of malignant tumors.

The vast majority of cancer-related morbidities and mortalities are due to an accumulation of genetic and epigenetic changes that result in the acquisition of the metastatic phenotype (1). It is estimated that this year alone, 1.2 million new cases of invasive cancer will be diagnosed leading to 550,000 cancer deaths (2). The formation of metastases is a complex, multistep process that requires the interplay of many genes, including those involved in cell survival, proliferation, morphogenesis, motility, invasion, and neovascularization (3). Mutations in the Ras family of GTP binding proteins represent one of the most commonly observed genetic alterations in human cancers (4). Although the precise role that Ras plays during the metastatic progression of human tumors remains unclear, oncogenic mutants of Ras are able to render both tumorigenic and metastatic phenotypes to a variety of murine cells (5). This allows for the study of the array of genetic and epigenetic events downstream of Ras signaling that mediate metastatic propensity (6).

The transmembrane Met receptor tyrosine kinase has been identified as the receptor for hepatocyte growth factor/scatter factor (HGF/SF; refs. 7 and 8). Under normal conditions, HGF/SF expression is usually limited to cells of mesenchymal origin and acts predominantly on epithelial and endothelial cells expressing the Met receptor to elicit cellular responses, including cellular proliferation, differentiation, motility, and invasion (reviewed in ref. 9). In addition to its role during normal physiological processes (10), several lines of evidence suggest that aberrant HGF/SF-Met signaling contributes to tumor development and progression to the malignant phenotype. Expression of mutant Met receptors that are activated by means of point mutations, or coexpression of both Met and HGF/SF, produces cells that are both tumorigenic and metastatic when injected into immune-compromised mice (11). In addition, there are numerous reports that Met and/or HGF/SF are overexpressed in a variety of human tumors, often in association with high tumor grade and poor prognosis (12).

Recently, we reported that ectopic expression of an activated allele of the *ras* oncogene (V12 H-Ras) induced significant expression of the Met receptor tyrosine kinase in C127 and NIH 3T3 cells, two murine cell lines that normally express low or negligible amounts of the receptor (13). This observation is not exclusive to rodent cell lines; introduction of activated *ras* into human thyroid epithelial cells also results in *met* overexpression (14). Though purely coincidental in nature, these data suggest that the Met proto-oncogene may play a role during Ras-mediated tumor development and/or metastasis. To directly assess the contribution of HGF/SF-Met signaling to both the tumorigenic and metastatic potential of *ras*-transformed cells, we constructed murine and human dominant-negative Met receptor mutants and used these to genetically block HGF/SF-Met signaling in *ras*-transformed cell lines. In this paper, we show that these mutants inhibit endogenous Met signaling, and that their expression suppresses Ras-mediated tumor growth and metastasis. As such, we propose that Met plays a prominent role during tumor cell growth and metastasis elicited by the *ras* oncogene.

Materials and Methods

Expression Plasmids. To construct the dominant-negative mouse Met, pMB11, a plasmid containing a full-length mouse *met* cDNA (15), was modified by two sequential rounds of site-directed mutagenesis following the QuikChange protocol (Stratagene). The first round of mutagenesis produced a L1108A (AAA → GCA) mutation and the second round produced the Y1305F (TAC → TTG) and Y1311F (TAT → TTG) mutations. The mutated *met* cDNA was transferred to the pcDNA6/His expression vector (Invitrogen) containing an additional C-terminal V5 epitope followed by six histidine residues yielding DN-mMet/pcDNA6. The presence of the indicated mutations and the 3′ junction of DN-mMet/pcDNA6 were verified by sequencing. To construct the dominant-negative human Met, a plasmid containing full-length human *met* cDNA (15) was modified by three sequential rounds of two-step PCR following standard protocols. The first PCR replaced the 21-aa carboxy terminus of human Met (TTG-TCA-TCA-GAA-GAT-AAC-GCT-GAT-GAT-GAG-GTG-GAC-ACA-CGA-CCA-GCC-TCC-TTC-TGG-GAG-ACA-TCA-TAG) with the 12 aa

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Abbreviations: HGF/SF, hepatocyte growth factor/scatter factor; DN-mMet, dominant-negative Met molecule; DN-hMet, dominant-negative human *met*.

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constituting the mouse Met C terminus (CCA-TCC-CAA-GAC-AAC-ATT-GAT-GGC-GAG-GGG-AAC-ACA-TGA), the second produced Y1367F (TAT → TTT) and Y1374F (TAT → TTG) mutations, and third produced K1110A (AAA → GCA) mutation. The final construct was sequence verified and the mutated *met* cDNA was transferred into the expression vector pRK5 (Becton Dickinson PharMingen) to produce hMet/pRK5.

Cell Lines. Mouse B16-F1 melanoma cells were obtained from ATCC and grown in DMEM media supplemented with 10% FBS. B16-F1 cells were cotransfected with pIRES-P (a generous gift of Dr. Steve Hobbs, Institute of Cancer Research, London) as a puromycin-selection plasmid and either pRK5 empty plasmid or DN-hMet/pRK5, using Lipofectamine reagent according to the manufacturer's instructions (Life Technologies, Gaithersburg, MD). B16-F1 cells were also transfected with pcDNA6 or DN-mMet/pcDNA6 by using the same procedure. Stable cell colonies expressing human or mouse DN-Met were selected by using 1 μg/ml puromycin or 3 μg/ml blasticidin, respectively. Parental and Ras-transformed C127 and NIH 3T3 (490) cells as well as their growth conditions and transfection procedures have been described (13). A clonal population of *ras*-transformed C127 cells was stably transfected with either pcDNA6 empty plasmid or the DN-mMet/pcDNA6 plasmid by using the DOTAP reagent (*N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium methylsulfate) according to the manufacturer's instructions (Roche Molecular Biochemicals). After ≈3 weeks of growth in media supplemented with 3 μg/ml blasticidin (Invitrogen), a number of single-cell colonies were expanded and cultured individually. To generate *ras*-transformed NIH 3T3 cells expressing dominant-negative human *met*, NIH 3T3 cells were cotransfected with either pDCR empty plasmid or pDCR V12 H-Ras (13) together with either the pRK5 empty plasmid or DN-hMet/pRK5 using the DOTAP procedure. After ≈2 weeks of growth in media supplemented with 400 μg/ml active Geneticin (G418; Roche), a number of single-cell clones were expanded and cultured individually.

Immunoprecipitation and Western Blotting. Western analysis and immunoprecipitation procedures for Met, phosphotyrosine (pY), and HA-tagged Ras have been described (13). Otherwise, nitrocellulose membranes were blocked for 1 h with 5% BSA and incubated overnight with a 1 μg/ml dilution of anti-V5 antibody (Invitrogen), anti-phospho-p44/42 mitogen-activated protein kinase (MAPK) (Cell Signaling Technologies, Beverly, MA), or anti-ERK1/ERK2 (Santa Cruz Biotechnology) in 1% BSA. Membranes were washed and incubated with horseradish peroxidase secondary antibody (0.2 μg/ml dilution) for 1 h before enhanced chemiluminescence (ECL) detection (Amersham Pharmacia).

Invasion Assays. Invasion of B16-F1 melanoma cells through basement membrane (Matrigel) coated filters was determined by resuspending cells in DMEM containing 0.1% BSA. B16-F1 cells (5×10^4) were applied to the upper side of a Matrigel-coated invasion chamber (Becton Dickinson) essentially as described (13). The chamber was placed in a 24-well culture plate containing DMEM/0.1% BSA with or without 50 ng/ml of HGF/SF. Chambers were incubated at 37°C for 4 days and noninvading cells were removed from the upper side of the filter by scraping. Invading cells were stained by using crystal violet, excess dye was removed by washing with water, and representative fields were photographed. Invasion of ras-transformed C127 cells followed similar procedure except the chambers were incubated at 37°C for 24 h and invading cells were stained using Diff-Quik staining solution (Baxter, Deerfield, IL).

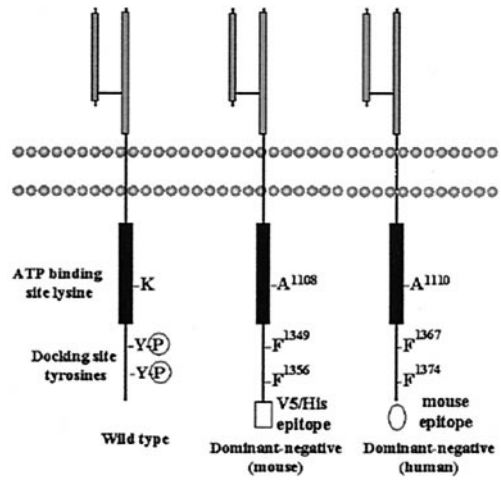

Fig. 1. Schematic of dominant-negative Met mutant receptors. Light and dark gray boxes designate the Met extracellular domain and the intracellular tyrosine kinase domain, respectively. A lysine critical for kinase activity was mutated to alanine and two tyrosines in the C-terminal region of the protein that are required for interactions with a number of effector proteins were mutated to phenylalanine. The mouse dominant-negative Met contains a C-terminal V5 epitope tag. The C-terminal 21 aa of the human dominant-negative Met were replaced by the C-terminal 12 aa of mouse Met.

***In Vivo* Tumorigenicity and Experimental Metastasis Assays.** Four-week-old female athymic nude mice (Ncr, nu/nu) were obtained from the Animal Production Area, National Cancer Institute–Frederick Cancer Research and Development Center, Frederick, MD. Animal care was provided in accordance with the procedures outlined in the *Guide for the Care and Use of Laboratory Animals*. Analysis of tumor cell growth and metastatic capabilities were preformed essentially as described (13). Briefly, trypsinized cells were resuspended at a final concentration of 1×10^6 cells/ml in HBSS and 0.1 ml of cell suspension was injected into either the s.c. region of the shoulder or the tail vein. Animals were killed when they appeared distressed or as indicated in the text. For histological examination, lungs were inflated and fixed in 10% neutral buffered formalin at necropsy, processed in paraffin, step sectioned at 5 μm, and stained with hematoxylin and eosin according to standard protocols (16).

Results

Construction of Dominant-Negative Met Mutants. Mutants of Met have recently been described that interfere with endogenous Met signaling (17). These mutant receptors contained either two C-terminal tyrosine residue mutations to prevent binding of downstream signaling molecules, or a deletion of the majority of the tyrosine kinase domain. We have generated dominant-negative Met mutant molecules that contain three point mutations within the otherwise wild-type Met receptor (Fig. 1). To inactivate the intrinsic kinase activity of the receptor, we mutated the essential lysine residue within the ATP-binding loop of the kinase domain (18). Also, to prevent recruitment of signaling complexes following receptor activation, we mutated two tyrosine resides in the receptor C terminus that when transphosphorylated mediate the binding of several downstream signaling molecules (reviewed in ref. 19). As such, we expected that following HGF/SF stimulation, the mutant Met receptors would be unable to transphosphorylate adjacent wild-type receptors. Additionally, wild-type receptors would be unable to transphosphorylate and activate the multisubstrate binding site of the mutant receptor because of the C-terminal tyrosine mutations. This triple point mutant would therefore form nonfunctional

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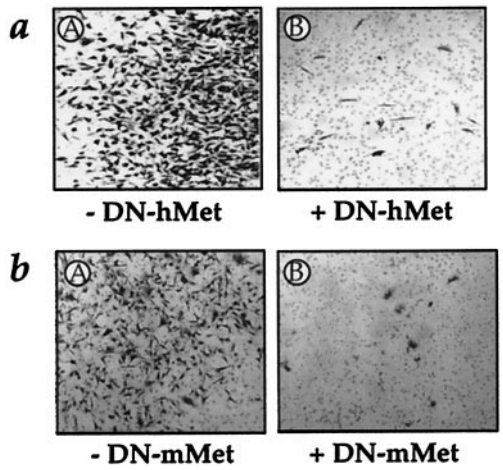

Fig. 2. Expression of DN-Met inhibits HGF/SF-mediated invasion of B16-F1 melanoma cells. (*a*) B16-F1 cells stably transfected with control vector (pRK5, *A*) or human dominant-negative Met (DN-hMet/pRK5, *B*) were placed onto the upper chamber of Matrigel filters and lowered into wells containing 50 ng/ml HGF/SF for 96 h. Invading cells adhering to the underside of the filter were stained and photographed. (*b*) B16-F1 cells stably transfected with control vector (pcDNA6, *A*) or mouse dominant-negative Met (DN-mMet/pcDNA6, *B*) and assayed as in *a*.

dimers with endogenous wild-type receptor, thereby preventing functional Met signaling.

During the construction of the human dominant-negative Met molecule, the last 21 amino acids of the human Met were replaced by the last 12 amino acids of mouse Met to distinguish this mutant molecule from endogenous receptors when expressed in human cells (Fig. 1). In addition, this allowed for the direct comparison in levels of expression between wild-type and mutant Met receptors when expressed in murine cell lines, using an antibody that recognizes the C terminus of murine Met. Because we were unsure whether a human dominant-negative Met molecule would efficiently inhibit endogenous murine Met in the mouse cell lines under study, we also constructed the equivalent mouse dominant-negative Met molecule (DN-mMet). To discriminate between the mutant murine receptor and the wild-type receptor, the murine Met mutant was engineered to contain a C-terminal V5 epitope tag (Fig. 1).

Validation of Dominant-Negative Met Mutant Receptors. During the validation of our dominant-negative Met mutants, we isolated numerous clones from different cell lines expressing either human or mouse mutant receptors. To initially determine whether the human and mouse mutant receptors could attenuate HGF/SF-Met signaling, mutant Met receptors were stably expressed in B16-F1 melanoma cells. Western blot analysis confirmed that several of these clones expressed dominant-negative human *met* (DN-hMet) and DN-mMet (data not shown). B16-F1 clones expressing the mutant receptors were then examined for their respective abilities to inhibit HGF/SF-mediated cell invasion *in vitro*. Ectopic expression of either DN-mMet or DN-hMet in B16-F1 cells completely inhibited the ability of these cells to invade Matrigel basement membrane in response to HGF/SF stimulation *in vitro* (Fig. 2 *a* and *b*). These data demonstrate that ectopic expression of either the human or mouse dominant-negative mutants is sufficient to attenuate the HGF/SF response in mouse cell lines.

Ras-transformed C127 and NIH 3T3 cells express higher levels of Met and are more responsive to HGF/SF simulation when compared with their nontransformed counterparts (13). Therefore, to address the role that Met plays in these cells we isolated (*i*) NIH 3T3 cell clones that stably expressed activated *ras* (V12 H-Ras) and increasing amounts of DN-hMet and (*ii*) C127 cell clones that stably expressed activated *ras* and increasing amounts of dominant-negative mouse *met* (DN-mMet) (Fig. 3 *a* and *b*). To determine whether these mutants were able to inhibit Met signaling in *ras*-transformed cells, we performed both biochemical and functional studies using *ras*-transformed C127 cells. *Ras*-transformed C127 cells were evaluated because they expresses higher levels of Met and do not produce endogenous HGF/SF, and are therefore significantly more responsive to exogenous HGF/SF stimulation when compared with *ras*-transformed NIH 3T3 cells (13). We stimulated *ras*-transformed C127 cells with or without ectopic expression of DN-mMet with 50 ng/ml HGF/SF. Met was immunoprecipitated with anti-mouse Met antibodies and the immune conjugates were analyzed by Western blotting using an antiphosphotyrosine antibody to determine the degree of receptor phosphorylation. As expected, addition of HGF/SF to control *ras*-transformed C127 cells (C127R) led to a significant increase in Met tyrosine phosphorylation (Fig. 3*c*, lanes 1 and 2). However, in cells that coexpressed DN-mMet, we observed an inverse correlation between the level of DN-mMet expression and the amount of Met tyrosine phosphorylation (Fig. 3*b* and 3*c*, lanes 3–8). These results demonstrate that expression of DN-mMet interferes with Met receptor phosphorylation in response to HGF/SF. We observed similar results when using B16-F1 cells expressing DN-hMet (data not shown). We next investigated activation of signaling pathways downstream of Met activation. In response to 50 ng/ml HGF/SF, control C127R cells displayed a robust increase in ERK2 phosphorylation (activation). In cells coexpressing DN-Met, this was reduced by 60% in three separate experiments (Fig. 3*d*). Importantly, expression of DN-Met had no effect on the basal ERK2 phosphorylation in *ras*-transformed cells (lanes 1 and 3).

In the absence of two of the C-terminal tyrosines, numerous other C-terminal tyrosines can be phosphorylated following HGF/SF stimulation (18, 20). Thus, Met was still phosphorylated to some degree despite a clear reduction in Met tyrosine phosphorylation in cells expressing high levels of DN-mMet (Fig. 3*c*, lanes 7 and 8). It was therefore necessary to test whether ectopic expression of the DN-Met isoforms could functionally block Met in *ras*-transformed cells, as observed in the B16-F1 melanoma cell line (Fig. 2). For this purpose, we performed *in vitro* invasion assays to determine whether cell invasion through basement membrane was effected by DN-Met expression following HGF/SF stimulation. We plated *ras*-transformed C127 control cells or cells coexpressing DN-mMet on the upper surface of Matrigel coated filters, stimulated these cells with 50 ng/ml HGF/SF, and recorded the degree of invasion after 22 h. As reported (13), *ras*-transformed C127 cells readily invaded and migrated through basement membrane in response to HGF/SF (Fig. 3*e*). In contrast, *ras*-transformed C127 cells expressing DN-mMet did not invade significantly following HGF/SF stimulation. This effect was not attributable to differential growth *in vitro*, because all clones were shown to proliferate at identical rates regardless of DN-Met expression (data not shown). Together these data demonstrate that the DN-Met mutant receptors suppress HGF/SF-Met signaling and inhibit HGF/SF-mediated invasion of *ras*-transformed cells *in vitro*.

Effect of Met Inhibition on Ras-Mediated Tumorigenesis and Metastasis. The major question that we wanted to address was whether inhibition of Met could influence *ras*-mediated tumorigenesis. We therefore injected *ras*-transformed NIH 3T3 control cells or cells coexpressing DN-hMet s.c. into the back of athymic nude mice and monitored tumor growth over time. Both *ras*-transformed NIH 3T3 control cells and cells expressing DN-hMet formed s.c. tumors (Fig. 4*a*). However, cells coexpressing DN-hMet formed tumors with somewhat delayed kinetics; after

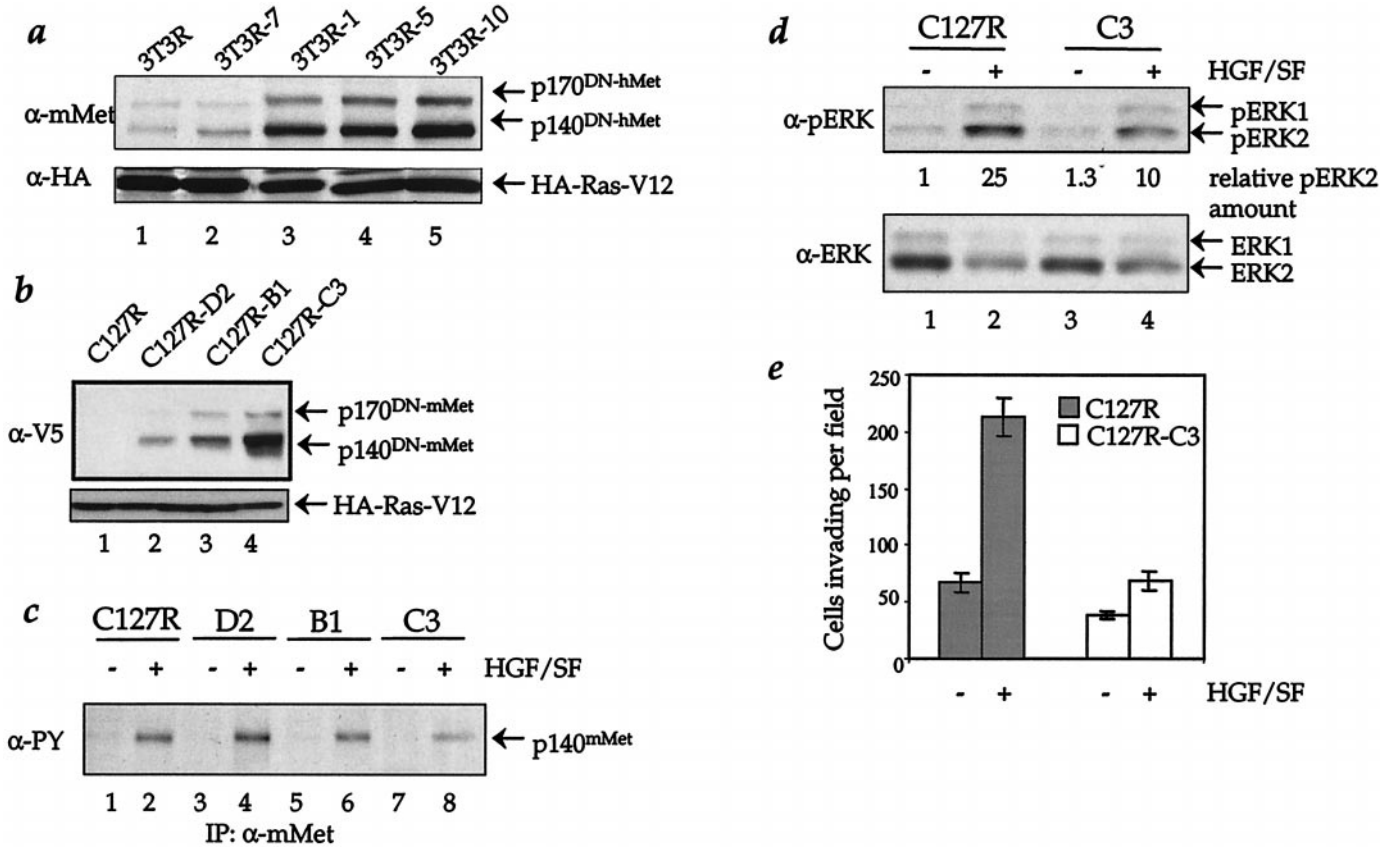

Fig. 3. Expression of DN-Met suppresses HGF/SF-mediated Met signaling in *ras*-transformed cells. (*a*) Cell extracts from NIH 3T3 cells stably transfected with V12 H-Ras and either control plasmid (lane 1, 3T3R) or a plasmid encoding dominant-negative human Met (lanes 2–5) were analyzed by Western blotting with anti-mouse Met (SP260) and anti-HA (Y-11) antibodies. Clone designations are shown above. (*b*) Cell extracts from *ras*-transformed C127 cells transfected with either control plasmid (lane 1, C127R) or a plasmid encoding dominant-negative mouse Met (lanes 2–4) were analyzed by Western blotting with anti-HA (Y-11) and anti-V5 antibodies. Clone designations are shown above. (*c*) The indicated C127 clones were mock treated (lanes 1, 3, 5, and 7) or treated with 50 ng/ml HGF/SF for 5 min (lanes 2, 4, 6, and 8). Met was precipitated from cell extracts by using anti-mouse Met antiserum (SP260), and the immunoprecipitates were assayed by Western blotting using antiphosphotyrosine antibodies (4G10). (*d*) The indicated C127 clones were mock treated (lanes 1 and 3) or treated with HGF/SF as in *c* (lanes 2 and 4). Cell extracts were assayed by Western blotting using anti-phospho-ERK1/ERK2 (E10) and anti-ERK1/ERK2 (SC-94/SC-154) antibodies. The degree of ERK2 phosphorylation was determined relative to total ERK2 levels by densitometry of Western blots using QUANTISCAN software. (*e*) Indicated *ras*-transformed C127 cells were placed onto the upper chamber of Matrigel filters and lowered into wells containing media with or without 50 ng/ml HGF/SF for 22 h. Invading cells adhering to the underside of the filter were stained and quantified by counting. Data represent mean cell number from triplicate experiments (±SE).

28 days the *ras*-transformed NIH 3T3 control cells formed tumors with an average volume of 324 mm^2, whereas *ras*-transformed NIH 3T3 cells expressing DN-hMet formed tumors with an average volume of 129 mm^2 (Fig. 4*a*). We observed similar results with s.c. injected *ras*-transformed C127 cells (Fig. 4*b*). After 20 days, the *ras*-transformed C127 cells formed tumors with an average size of 2078 mm^3, whereas tumors derived from cells coexpressing DN-mMet averaged 1326 mm^3. Despite this delay, tumor growth proceeded in mice injected with cells harboring DN-Met to the point where animal sacrifice was required. Therefore, expression of either human or mouse dominant-negative Met mutants in two different murine cell lines results in a delay in *ras*-mediated tumor development, but not complete inhibition of tumor growth. These data suggest that *ras* and Met may cooperate during the early stages of tumor development.

We next determined whether expression of DN-Met could modulate Ras-mediated metastasis. We performed experimental metastasis assays and injected *ras*-transformed NIH 3T3 control cells or cells coexpressing DN-hMet into the tail vein of athymic nude mice. After 4 weeks the animals were killed and examined for the presence of lung metastases (Table 1). Eight of ten mice injected with control *ras*-transformed NIH 3T3 cells formed visible macroscopic lung metastases after 4 weeks. In contrast, only one mouse of fifteen injected with NIH 3T3 cells expressing high levels of DN-hMet displayed a single metastatic lung lesion. Similar effects were observed in mice injected with *ras*-transformed C127 cells (Table 2). Although equivalent numbers of mice injected with the *ras*-transformed control cells or with cells coexpressing DN-mMet exhibited lung metastases, there was a 4-fold decrease in the number of metastases in the latter (Fig. 4*c*, Table 2). We also observed a possible dose-dependent effect: mice injected with *ras*-transformed C127 cells that expressed intermediate levels of DN-mMet formed corresponding numbers of lung metastases (Table 2). The lungs were weighed as an independent measure of lung tumor burden. Lungs from mice injected with *ras*-transformed control C127 cells weighed on average 0.53 ± 0.17 g, whereas lungs from mice expressing high levels of DN-mMet weighed on average 0.29 ± 0.03 g, representing a 55% reduction in tumor burden. These data demonstrate that inhibition of Met can suppress Ras-mediated experimental lung metastasis.

Discussion

We have previously shown that overexpression of the Met receptor tyrosine kinase accompanies *ras*-mediated cellular

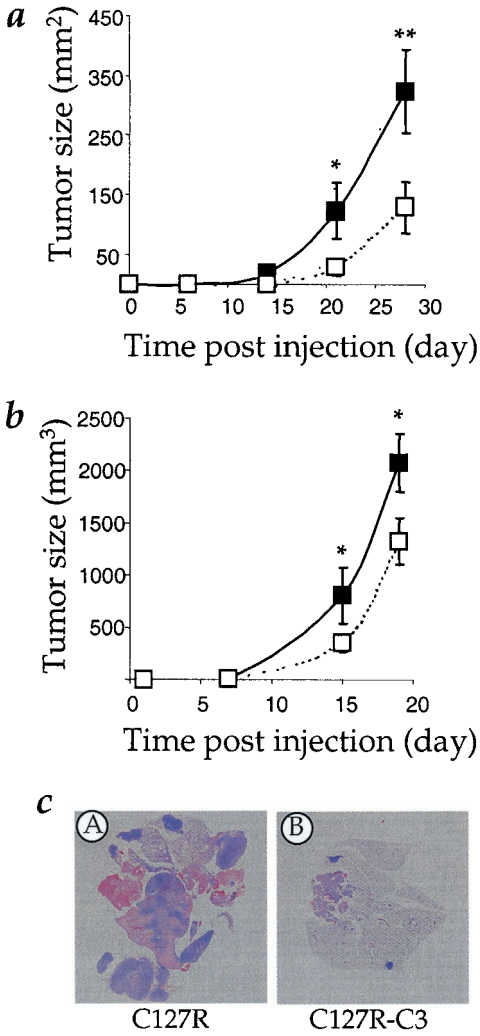

Fig. 4. Effect of dominant-negative Met expression on the growth and metastasis of *ras*-transformed cells. (*a*) *ras*-transformed NIH 3T3 control cells (clone 3T3R, solid line) or cells expressing dominant-negative human Met (clone 3T3R-10, hashed line) were injected s.c. into the back of athymic nude female mice and the size of the resulting tumors measured at the indicated times. Data represent mean tumor area (±SE) from four mice. A one-tailed *t* test was used to determine the statistical difference between the tumors with * designating $P \leq 0.09$ and ** designating $P \leq 0.02$. (*b*) *ras*-transformed C127 control cells (clone C127R, solid line) or cells expressing dominant-negative murine Met (clone C127R-C3, hashed line) were injected and analyzed as described in *a*. Data represent mean tumor volume (±SE) from five mice with * designating $P \leq 0.09$ computed as in *a*. (*c*) Photographs of representative hematoxylin and eosin stained lung sections obtained 4 weeks post tail vein injection of *ras*-transformed C127 control cells (C127R) or cells expressing dominant-negative mouse Met (C127R-C3). Tumor lesions appear as intense blue staining.

transformation, suggesting that Met may be involved in *ras*-mediated tumor progression (13, 14). Recent studies have suggested that stimulation of the Ras-Raf-mitogen-activated protein kinase (MAPK) pathway induces Met expression through activation of the AP-1, SP-1, and possibly Ets transcription factors (21–23). To directly assess whether Met signaling contributes to Ras-mediated tumorigenicity and metastasis, we set out to genetically inhibit endogenous Met signaling through the ectopic expression of dominant-negative Met receptor mutants. Dominant-negative receptors have been proposed to interfere with wild-type receptor activation either by forming inactive heterodimers (24) or by sequestering ligand or intracellular

Table 1. DN-hMet inhibits metastasis of ras-transformed NIH3T3 cells

Cells injected*	DN-hMet expression	$n^{\dagger}$ (%)	Mean number metastasis per lung
3T3R/3T3R-7	None/low	8/10 (80)	6
3T3R-1	High	0/4 (0)	0
3T3R-5	High	0/4 (0)	0
3T3R-10	High	1/7 (14)‡	0

*Cells were injected into the tail vein of athymic nude mice and metastasis scored after 4 weeks or when the mice appeared distressed.
†Number of mice with lung metastasis per number of mice injected.
‡One mouse was found to exhibit a single metastasis in the lung.

signaling components (25). Our data show that expression of either human or mouse Met mutants that contain both kinase domain and multisubstrate binding site mutations (Fig. 1) can suppress HGF/SF-mediated Met signaling to an extent sufficient to abrogate Met function *in vitro* (Figs. 2 and 3). Although we cannot completely eliminate the possibility that our dominant-negative mutants titrate other intracellular signaling molecules, expression of DN-Met had no effect on basal (HGF/SF-independent) activation of ERK2 (Fig. 3*d*) or AKT (data not shown). These particular Met mutants will prove valuable tools for determining the precise role for Met during various biological phenomena both *in vitro* and *in vivo*.

The results presented in this paper demonstrate that Met signaling contributes to tumor growth and metastasis elicited by the *ras* oncogene. Despite identical growth rates *in vitro*, *ras*-transformed cell lines that express either mouse or human dominant-negative Met mutants display delayed kinetics of s.c. tumor growth when compared with control cells (Fig. 4 *a* and *b*). Nonetheless, cells expressing DN-Met did form significant tumor burden in mice following this delay. These data may suggest that Met cooperates with the *ras* oncogene during the early stages of tumor development, possibly through its ability to mediate local tumor cell invasion, proliferation, and/or survival. The precise role that Met plays during Ras-mediated tumorigenesis is an important area for future study. To address the issue of whether Met signaling is required for *ras*-mediated metastasis, *ras*-transformed cells coexpressing dominant-negative Met mutants were injected into the tail vein of athymic nude mice and assessed for their abilities to form lung metastases. Our results clearly show that expression of dominant-negative Met mutants in *ras*-transformed C127 and NIH 3T3 cells significantly suppresses lung metastasis. The experimental metastasis assay used during the course of these experiments recapitulates the later stages of the metastatic cascade, that is extravasation, colonization, and growth within lung tissue (26). Thus, it appears that Met plays a crucial role in some/all of these later stages of metastasis, which represent the most likely opportunity for clinical intervention.

Table 2. DN-mMet inhibits metastasis of ras-transformed C127 cells

Cells injected	DN-mMet expression	n (%)	Mean number metastasis per lung
C127R	None	5/9 (56)	8
C127R-D2	Low	3/5 (60)	4*
C127R-C3	High	6/10 (60)	2**

Columns are as in Table 1. *$P \leq 0.06$ or **$P \leq 0.03$ for a one-tailed *t* test between the control and sample population means.

On the basis of these results we propose that during tumor progression, acquisition of an oncogenic *ras* mutation leads to elevated Met expression, which on activation can result in metastatic disease. Thus, human tumors harboring oncogenic forms of *ras* may promote metastasis through increased expression of the Met receptor. Increased expression of Met may in turn, mediate metastasis through either ligand-dependent or -independent mechanisms (27–29). In some circumstances, the tumor cell may express endogenous HGF/SF, as is the case with NIH 3T3 cells, such that activation of Met occurs in an autocrine fashion. This may be the mechanism by which certain classes of sarcomas harboring *ras* mutations metastasize, because these are typically autocrine for HGF/SF-Met activation (30, 31). In addition, paracrine release of HGF/SF from host stromal tissue is likely to be required to promote metastasis in Ras-transformed cells, such as C127 cells and the majority of human carcinoma cells, which typically do not produce endogenous HGF/SF. In these cases, stromal-derived HGF/SF is likely an essential component for the development of metastases. It is interesting to note that two organs frequently associated with high incidence of metastatic growth, the liver and lungs, express significant levels of HGF/SF (32). In addition, these data may help explain the failure of many human cell lines to metastasize in mice. Mouse HGF/SF fails to activate human Met (15), and as such there may be insufficient paracrine HGF/SF-Met activation to allow human cells to efficiently form metastases within mouse tissues.

To date, we have provided three lines of evidence to indicate that aberrant Met signaling contributes to Ras-mediated tumorigenesis and metastasis: (*i*) expression of activated *ras* in rodent cell lines leads to increased Met expression and renders them HGF/SF-responsive (13), (*ii*) Met-HGF/SF expression increases in Ras-mediated tumors and metastases (13), and (*iii*) expression of dominant-negative Met receptor mutants in Ras-transformed tumors suppresses their tumorigenic and metastatic potential. Numerous human carcinomas have a high incidence of Ras mutations, many of which also express aberrant levels of Met and/or HGF/SF (12, 33). For example, pancreatic carcinomas are associated with a high incidence of Ras mutations and a similar frequency of Met overexpression, correlated with metastatic potential and poor patient prognosis (34, 35). The possible relationship that exists between Ras and Met in these highly invasive tumors and the role of Met signaling in the etiology and progression of these and other tumor types certainly require further evaluation. We are currently investigating the events downstream of Met that may be important regulators of tumorigenicity and metastasis, and are also evaluating possible therapeutics that target HGF/SF-Met signaling, including neutralizing antibodies, small peptide inhibitors (36), and small molecule inhibitors (37, 38), as future treatments for metastatic disease.

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