

# RNA Interference Reveals that Ligand-Independent Met Activity Is Required for Tumor Cell Signaling and Survival

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## ABSTRACT

Hepatocyte growth factor/scatter factor-Met signaling has been implicated in tumor growth, invasion, and metastasis. Suppression of this signaling pathway by targeting the Met protein tyrosine kinase may be an ideal strategy for suppressing malignant tumor growth. Using RNA interference technology and adenovirus vectors carrying small-interfering RNA constructs (Ad Met small-interfering RNA) directed against mouse, canine, and human Met, we can knock down *c-met* mRNA. We show a dramatic dependence on Met in both ligand-dependent and ligand-independent mouse, canine, and human tumor cell lines. Mouse mammary tumor (DA3) cells and Met-transformed NIH3T3 (M114) cells, as well as both human and canine prostate cancer (PC-3 and TR6LM, human sarcoma (SK-LMS-1), glioblastoma (DBTRG), and gastric cancer (MKN45) cells, all display a dramatic reduction of Met expression after infection with Ad Met small-interfering RNA. In these cells, we observe suppression of tumor cell growth and viability *in vitro* as well as inhibition of hepatocyte growth factor/scatter factor-mediated scattering and invasion *in vitro*, whether Met activation was ligand dependent or not. Importantly, Ad Met small-interfering RNA led to apoptotic cell death in many of the tumor cell lines, especially DA3 and MKN45, but did not adversely affect MDCK canine kidney cells. Met small-interfering RNA also abrogated downstream Met signaling to molecules such as Akt and p44/42 mitogen-activated protein kinase. We further show that intratumoral infection with *c-met* small-interfering RNA adenovirus results in a substantial reduction in tumor growth. Thus, Met small-interfering RNA adenoviruses are reliable tools for studying Met function and raise the possibility of their application for cancer therapy.

## INTRODUCTION

Hepatocyte growth factor/scatter factor (HGF/SF) is a pleiotropic factor that induces many cellular functions including proliferation, migration, angiogenesis, and morphogenesis (1, 2). Met, the receptor for the HGF/SF ligand, mediates signaling to downstream molecules (1, 3, 4). HGF/SF is produced mainly by mesenchymal cells, whereas Met is preferentially expressed in epithelial and endothelial cells. In many types of tumor cells, Met signaling is activated through ligand-dependent autocrine or paracrine mechanisms (5, 6) or by ligand-independent activation (7). Enhanced signal transduction via the stimulation of this receptor contributes to the malignant phenotype (1).

HGF/SF binding to Met activates signaling downstream (8) through various pathways (1), such as the Ras mitogen-activated protein kinase (MAPK) pathways through Grb2-SOS complex formation or the Ras and Rac pathways responsible for tubulo-morphogenesis and cell spreading/actin reorganization. Likewise, STAT3 activation is required for HGF/SF-Met-mediated growth in soft agar and tumori-

genesis (9), and phosphatidylinositol 3'-kinase activation followed by Akt phosphorylation contributes to the prevention of apoptotic cell death (10,11). In addition HGF/SF-Met signaling can up-regulate the production of matrix metalloproteinases and urokinase that induce the degradation of extracellular matrices and basement membrane and enhance tumor invasion and metastasis (12, 13). Moreover, the activation of Met protein is involved in the induction of blood vessel formation in tumors by increasing the production of vascular endothelial growth factor (14, 15) and by simultaneously downregulating the antiangiogenesis factor thrombospondin-1 (16).

Since HGF/SF-Met signaling is implicated in many cancers<sup>4</sup> and regulates biological activities that contribute to the malignancy of tumor cells, targeting the Met receptor has become a matter of interest in the field of cancer biology. HGF/SF-neutralizing antibodies (17), *c-met* antisense oligonucleotides (18), dominant-negative forms of the Met protein (19, 20), ribozymes that target Met mRNA (21, 22), and small molecule Met tyrosine kinase inhibitor (23) are being investigated as possible strategies to block Met activation and suppress tumor growth, invasion, and metastasis.

RNA interference (RNAi) has developed into a new gateway for elucidating gene function. RNAi is a sequence-specific, posttranscriptional, gene-silencing mechanism that is effected through double-stranded RNA molecules homologous to the sequence of the target gene (24–26). Fragments of the double-stranded RNA called small-interfering RNAs can rapidly induce loss of function, and only a few molecules are required in a cell to produce the effect (25), which results from hybrid formation between mRNA and the homologous small-interfering RNA (siRNA; ref. 27). DNA vector-mediated RNAi technology has made it possible to develop therapeutic applications of this technology in mammalian cells (28–30). Several examples using retroviral (31–34) or adenoviral vector systems (35) to deliver siRNA for stable or transient expression, respectively, have been reported.

To study the key role of Met *in vitro* and *in vivo* in tumor cells, we produced adenovirus vectors encoding siRNA sequences directed against either mouse, canine, or human Met and under the control of the U6 promoter. We show that RNAi driven by these adenovirus constructs can effectively silence *met* RNA and protein expression in all ligand-dependent and ligand-independent mouse, canine, and human cell types tested. Functional analyses revealed that the abrogation of Met not only strongly inhibits proliferation, scattering, and migration but also induces apoptosis and suppresses tumorigenicity. These results show that *c-met* siRNA adenoviral vectors are a promising tool for the analysis of HGF/SF-Met function as well as potential vectors for targeting Met-expressing cancers.

## MATERIALS AND METHODS

**Cell Lines.** DA3 cells (poorly differentiated mouse mammary adenocarcinoma), M114 cells (NIH3T3 cells stably transfected with mouse met and mouse HGF/SF), SK-LMS-1 human leiomyosarcoma cells, PC-3 human prostate cancer cells, DBTRG human glioblastoma cells, Madin-Darby canine kidney (MDCK) cells, and TR6LM cells, and canine prostate carcinoma cell

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<sup>4</sup> <http://www.vai.org/metandcancer/>.

lines established from a spontaneous lung metastasis were grown in DMEM (Life Technologies, Inc., Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT). MKN45 human gastric cancer cells were grown in RPMI 1640 (Life Technologies, Inc., Invitrogen) supplemented with 10% fetal bovine serum.

**Met siRNA Expression Plasmids.** The mU6 pro vector containing the mouse U6 promoter (36) or the pSilencer 1.0-U6 siRNA expression vector (Ambion, Austin, TX) was used for the construction of mouse and human Met siRNA expression plasmids. The siRNA target finder and design tool provided by Ambion was used for selecting the siRNA sequences (see Table 1 footnote). We selected four mouse, three canine, and three human candidate siRNA sequences from Met mRNA sequences (Table 1) to minimize possible non-specific reactivity with target sequences. Moreover, these sequences also survived a BLAST search to exclude coding sequences with 17 or more contiguous base pairs of homology to other genes. The oligonucleotides that encode the Met mRNA 19-mer hairpin sequences were cloned into an expression vector plasmid (the *Bbs*I and *Xba*I sites in the mU6 pro vector and the *Apa*I and *Eco*RI sites in the pSilencer 1.0-U6 vector) and tested for Met suppression activity in either mouse or human cells, respectively.

**Construction of c-met siRNA Adenoviruses (Ad Met siRNA).** For the construction of recombinant adenoviruses, the AdEasy Adenoviral Vector System (human adenovirus serotype 5, or Ad5; Stratagene, La Jolla, CA) was used. First, we recloned the selected siRNA sequences with the U6 promoter into a pShuttle vector. As a mock vector, U6 promoter without the siRNA sequence was used. Then, pShuttle vectors containing siRNA sequences were linearized with *Pme*I and cotransformed with pAdEasy-1 into BJ5183 cells by electroporation. Positive (homologously recombined) clones were selected and confirmed by *Pac*I digestion. Adenoviral DNA plasmids with the correct insert were transformed into TOP10-competent cells (Invitrogen, Carlsbad, CA) and amplified. The linearized adenoviral DNA was prepared by digesting the plasmid with *Pac*I, after which it was transfected into the packaging cell line HEK293. Transfected cells were cultured for 7 days, and the virus was harvested. After repeating one more amplification cycle, large-scale amplification was done by with Cell Factory tissue culture plates (Nunc, Nalge Nunc International, Rochester, NY). Purification of the virus was done either by CsCl ultracentrifugation or by PEG8000 precipitation. The titer of the virus was evaluated through endpoint dilution.

**Adenovirus Infection.** Cells at 75 to 80% confluency were infected with Ad Met siRNA diluted in a small volume of growth medium containing 10% fetal bovine serum at a multiplicity of infection (m.o.i.) of 10 to 100 for 4 hours at 37°C. After 4 hours, fresh complete growth medium was added, and the cells were cultured in a CO<sub>2</sub> incubator at 37°C. After 2 to 4 days in culture, the adenovirus-infected cells were collected for Western blotting, proliferation assays, invasion assays, or morphologic analyses.

**Western Blot Analysis.** Cell extracts were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Invitrogen). The membranes were incubated with antibodies against Met (SP260: sc-162, Santa Cruz Biotechnology, Santa Cruz, CA; C-28: sc-161, Santa Cruz Biotechnology; 25H2: Cell Signaling, Beverly, MA); phospho-Met (Tyr1234/1235 rabbit polyclonal antibodies, Cell Signaling); p44/42 MAPK (rabbit polyclonal antibodies, Cell Signaling); phospho-p44/42 MAPK (Thr202/Tyr204 rabbit polyclonal antibodies, Cell Signaling); phospho-Akt (Ser473, 587F11, Cell Signaling); or  $\beta$ -actin (AC-15: ab6276, Abcam, Cambridge, MA) followed by horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotech-

nology). After incubation with ECL reagent (Amersham Biosciences, Buckinghamshire, United Kingdom), chemiluminescence signals were photographed and quantitated by image analysis.

**Reverse Transcription-PCR Analysis.** Total RNA was isolated with TRIzol reagent (Invitrogen). Reverse transcription was done with 1  $\mu$ g RNA and the SuperScript II RNase H<sup>-</sup> Reverse Transcriptase (Invitrogen). One microliter of the reverse transcriptase product was used for amplification of *c-met* or  $\beta$ -actin genes. The primers used were *met*-sense, 5'-AGCCAGTA-ATGATCTCAATAG-3'; *met*-antisense, 5'-TCAGGATAGGGGACAGGT-3';  $\beta$ -actin sense, 5'-CGTGACATCAAAGAGAAGCTGTG-3'; and  $\beta$ -actin antisense, 5'-GCTCAGGAGGAGCAATGATCTTGA-3'. The PCR conditions were 95°C for 5 minutes, followed by 35 cycles of 95°C, 1 minutes; 55°C, 1 minutes; and 72°C, 1 minute. The final extension was 72°C for 5 minutes.

**Scatter Assay.** Cells were seeded into six-well culture plates and treated with low-serum DMEM for 24 hours. Then assay medium containing HGF/SF (25 ng/mL) was added to the cells, and they were incubated overnight. Cell scattering was observed under phase contrast microscopy.

**Terminal Deoxynucleotidyl Transferase (TdT)-Mediated dUTP Nick End-Labeling (TUNEL) Assay.** In Situ Cell Death Detection Kit, POD (Roche Diagnostics, Mannheim, Germany) was used for the TUNEL technology. Cells (1,000/well) were seeded into 96-well microtiter plates. After the cells adhered to the bottom, they were infected with mU6-Ad5 or Ad Met siRNA viruses at a m.o.i. of 10, 50, or 100. Three and 6 days after infection, cells were fixed with 4% buffered formalin and processed for TUNEL assay according to the instruction manual. TdT reaction was done for 60 minutes at 37°C in a humidified atmosphere. Peroxidase substrate kit DAB (Vector Laboratories, CA) was used for the color development.

**Sub-G<sub>1</sub> Fraction Analysis.** Cells (5  $\times$  10<sup>4</sup>/well) were seeded into 6-well plates and infected with Ad Met siRNA at different m.o.i. (10, 50, or 100). Three and 6 days after infection, cells were harvested and processed for flow cytometric analysis. The suspensions of tumor cells were prepared with the detergent-trypsin method and then were stained with propidium iodide. Measurement of DNA cellular contents was done with a FACSCalibur flowcytometer (Becton Dickinson, Franklin Lakes, NJ). Fractions showing the DNA content <2C peak (namely, sub-G<sub>1</sub> fraction) were calculated with the CELLQuest software package and taken as apoptotic fractions.

**Tumorigenicity and Tumor Growth.** DA3 cells (1  $\times$  10<sup>5</sup>) were inoculated subcutaneously into the right flank of BALB/c mice. At 3 and 7 days, Ad Met siRNA (4  $\times$  10<sup>7</sup> infectious unit in 0.1 mL) was injected directly into the tumor. Tumor size was measured for 24 days after tumor inoculation. The mean and standard deviation were calculated for each data group, and the statistical difference was calculated with Student's *t* test.

## RESULTS

**Ad Met siRNA and Met Expression.** We produced four Ad Met siRNA viruses for mouse, three for human, and three for canine (Table 1). In addition, we constructed a control virus containing the mU6 promoter lacking siRNA sequences (mU6-Ad5).

M114, mouse NIH3T3 cells transformed with mouse *met* and HGF/SF, were infected with four different mouse Ad Met siRNA viruses (si-mMet-Ad5<sup>57, 60, 110, 178</sup>; Table 1) at m.o.i. 10, 50, and 100.

Table 1 *c-met* siRNA adenovirus constructs

Species	Name of adenovirus construct	siRNA gene position in <i>c-met</i> mRNA	19-mer target sequence
Mouse	si-mMet-Ad5 <sup>57</sup>	950	GCCGCGTATGTCAGTAAAC
	si-mMet-Ad5 <sup>60</sup>	988	GCAAATAGGAGCTAGCCCT
	si-mMet-Ad5 <sup>110</sup>	1839	GCGAGAGCAGACAAATAC
	si-mMet-Ad5 <sup>178</sup>	2762	GTGATCGTTCAACCGGATC
	si-hMet-Ad5 <sup>16</sup>	415	GACCTCAGAAGGTTGCTG
Human	si-hMet-Ad5 <sup>62</sup>	1236	GCCAGATTCTGCCGAACCA
	si-hMet-Ad5 <sup>221</sup>	3310	GTGCAGTATCCTCTGACAG
	si-dMet-Ad5 <sup>111</sup>	1904	GTGAGAGCAACAATAT
Dog	si-dMet-Ad5 <sup>197</sup>	2827	GTAATAGTTCAACCAGATC
	si-dMet-Ad5 <sup>222</sup>	3130	GTACAAATATCCTCTGACGG
	mU6-Ad5 (controlvirus)		

NOTE. The siRNA target finder and design tool is available on the following web site: [http://www.ambion.com/techlib/misc/siRNA\\_finder.html](http://www.ambion.com/techlib/misc/siRNA_finder.html). Abbreviation: siRNA, small-interfering RNA.

Met expression was determined on day 3 after infection by Western blot (Fig. 1A). The expression of Met protein was dramatically suppressed by si-mMet-Ad5<sup>57, 178</sup> at m.o.i. 50 and 100; Control mU6-Ad5 virus infected cells showed no effect on Met expression. Both the p140 and p170 forms of Met in M114 cells were reduced by si-mMet-Ad5 adenoviruses.

We tested the effect of human Ad Met siRNA viruses (si-hMet-Ad5<sup>16, 62, 221</sup>) on human DBTRG glioblastoma cells, PC-3 prostate cancer cells, and MKN45 human gastric carcinoma cells, all of which express high levels of Met protein. All three si-hMet-Ad5 viruses suppressed Met expression (Fig. 1, B–D), and the RNAi effect was strongest with si-hMet-Ad5<sup>221</sup>. Met is activated in PC-3 cells, and two si-hMet-Ad5<sup>62, 221</sup> viruses significantly suppressed Met expression. When PC-3 cells were infected with a mixture of all three forms (si-hMet-Ad5<sup>16, 62, 221</sup>) at 33 m.o.i. each, the RNAi effect was similar to the effect observed with si-hMet-Ad5<sup>221</sup> alone at 100 m.o.i. (Fig. 1C, left). MKN45 cells express very high levels of Met, and like PC-3 cells, Met appears to be activated in a ligand-independent fashion (data not shown). Again Met expression in MKN45 cells was dramatically suppressed by si-hMet-Ad5<sup>221</sup> at m.o.i. 50 to 100 (Fig. 1D).

**Specificity of Ad Met siRNA and RNAi Persistence after Cell Passage.** To test the specificity of the Ad Met siRNA, we infected DA3 mouse mammary adenocarcinoma cells and MKN45 human gastric cancer cells with siRNA adenoviruses directed against either mouse Met (si-mMet-Ad5<sup>178</sup>) or human Met (si-hMet-Ad5<sup>221</sup>) and

determined Met expression after 3 days (Fig. 2A). In DA3 cells, Met expression was only suppressed in the cells infected with mouse si-mMet-Ad5<sup>178</sup>, although si-hMet-Ad5<sup>221</sup> virus effectively suppressed human Met expression in human cells (Fig. 1, B–D). Conversely, Met expression in MKN45 cells was only suppressed by si-hMet-Ad5<sup>221</sup>, and no Met reduction was observed in si-mMet-Ad5<sup>178</sup>-infected cells. These results indicate that Met RNAi works in a sequence-specific manner. The RNAi effect of si-mMet-Ad5 adenovirus occurs at the mRNA level (Fig. 2B). Dramatic reduction in *met* mRNA expression was observed by reverse transcription-PCR in DA3 cells infected with si-mMet-Ad5<sup>178</sup> virus. By contrast, no reduction in *met* mRNA was observed in control mU6-Ad5 virus-infected cells.

Next, we asked how long the RNAi effect continues after cell passage. M114 cells were infected with si-mMet-Ad5<sup>178</sup> at m.o.i. 100, and Met expression was determined at 3-day intervals for up to 9 days (Fig. 2C, left). Three days after infection, p140 and p170 Met expression was <5% of the noninfected or cells infected with control mU6-Ad5 virus. Moreover, when cells were trypsinized and replated at lower density after culture for 3 days, Met expression was still strongly suppressed (*Passage 1*, Fig. 2C, left). Even after a second passage, Met expression was still markedly reduced. These results show that not only is the Met expression suppressed in the primary infected cells, but the RNAi effect persists for several cell passages. We tested the human leiomyosarcoma tumor cell line SK-LMS-1 cells

Fig. 1. The effect of Ad Met siRNA infection on Met expression in tumor cells. **A.** M114 mouse *met*-transformed NIH3T3 cells were infected with four different Ad Met siRNA at m.o.i. of 10, 50, and 100. After 3 days, cells were harvested, and Met expression level was determined by Western blot. Twenty micrograms of protein was loaded in each lane. Met expression was suppressed by si-mMet-Ad5<sup>57</sup> and si-mMet-Ad5<sup>178</sup> at m.o.i. 50 and 100. No significant reduction in Met expression was observed in control (mU6 promoter only) virus. The strongest RNAi effect was from si-mMet-Ad5<sup>178</sup>. **B.** DBTRG human glioblastoma cells were infected with three different constructs of Ad Met siRNA at different m.o.i. (0, 10, 50, and 100). After 3 days, cells were harvested, and Met expression level was observed by Western blot. Twenty micrograms of protein was loaded in each lane. Met expression was dramatically suppressed by si-hMet-Ad5 numbers 16, 62, and 221 at m.o.i. 10 to 100, and si-hMet-Ad5<sup>221</sup> showed the strongest RNAi effect. No reduction in Met expression was observed with control mU6-Ad5 virus. **C. Left:** PC-3 human prostate cancer cells were infected with si-hMet-Ad5 viruses at m.o.i. 100. After 3 days, cells were harvested, and the Met expression level was observed by Western blot. Thirty micrograms of protein was loaded in each lane. Again, si-hMet-Ad5<sup>221</sup> showed the strongest RNAi effect. A mixture of all three (si-hMet-Ad5<sup>16, 62, 221</sup>) at m.o.i. 33.3 each gave a similar effect. **Right:** PC-3 cells were infected with control (mU6-Ad5) or *c-met* siRNA (si-hMet-Ad5<sup>221</sup>) adenoviruses at different m.o.i. (0, 10, 50, and 100). After 3 days, cells were harvested, and the Met expression level was observed by Western blot. Thirty micrograms of protein was loaded in each lane. Met expression was suppressed by si-hMet-Ad5<sup>221</sup> dose dependently. **D.** MKN45 human gastric cancer cells were infected with control (mU6-Ad5) or *c-met* siRNA (si-hMet-Ad5<sup>221</sup>) adenoviruses at different m.o.i. (0, 10, 50, and 100). After 3 days, cells were harvested, and the Met expression level was observed by Western blot. Thirty micrograms of protein was loaded in each lane. This cell line expresses a high level of Met. Again, Met expression was dramatically suppressed by si-hMet-Ad5<sup>221</sup> at m.o.i. 50 to 100.

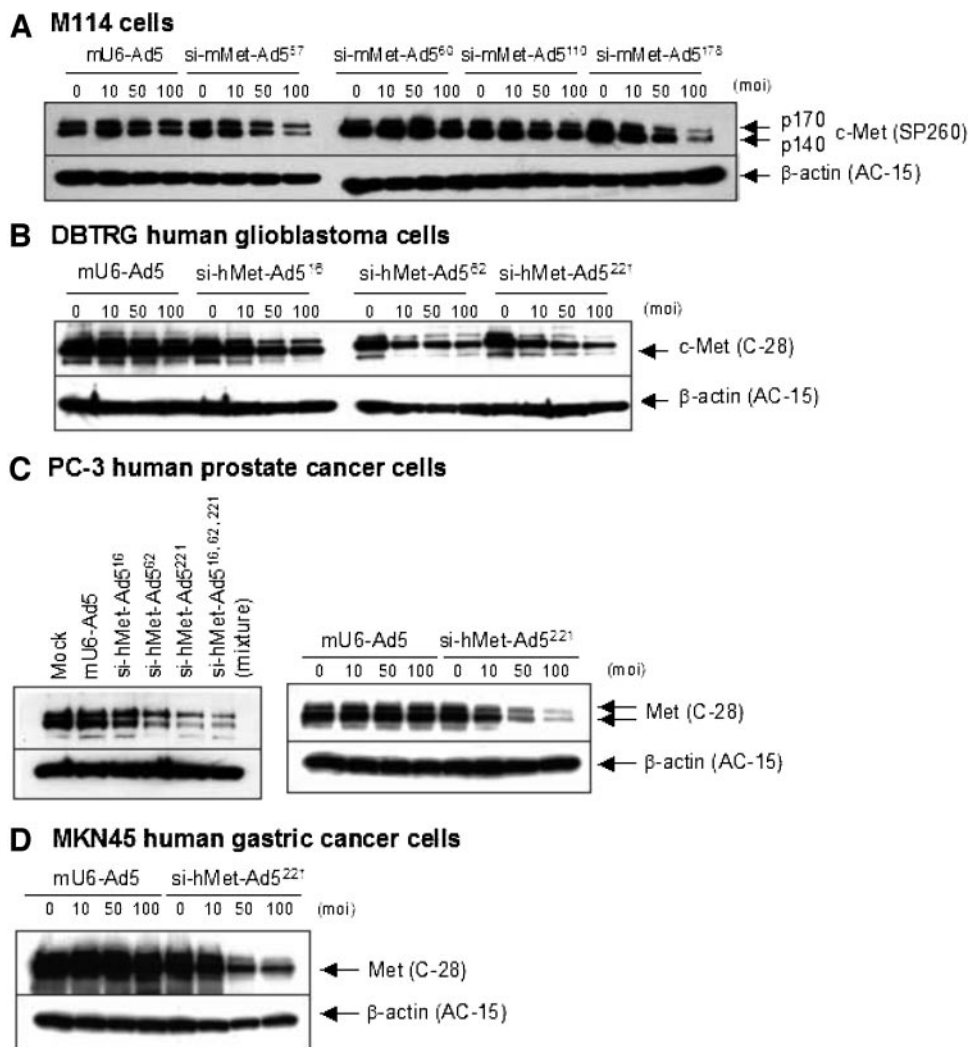
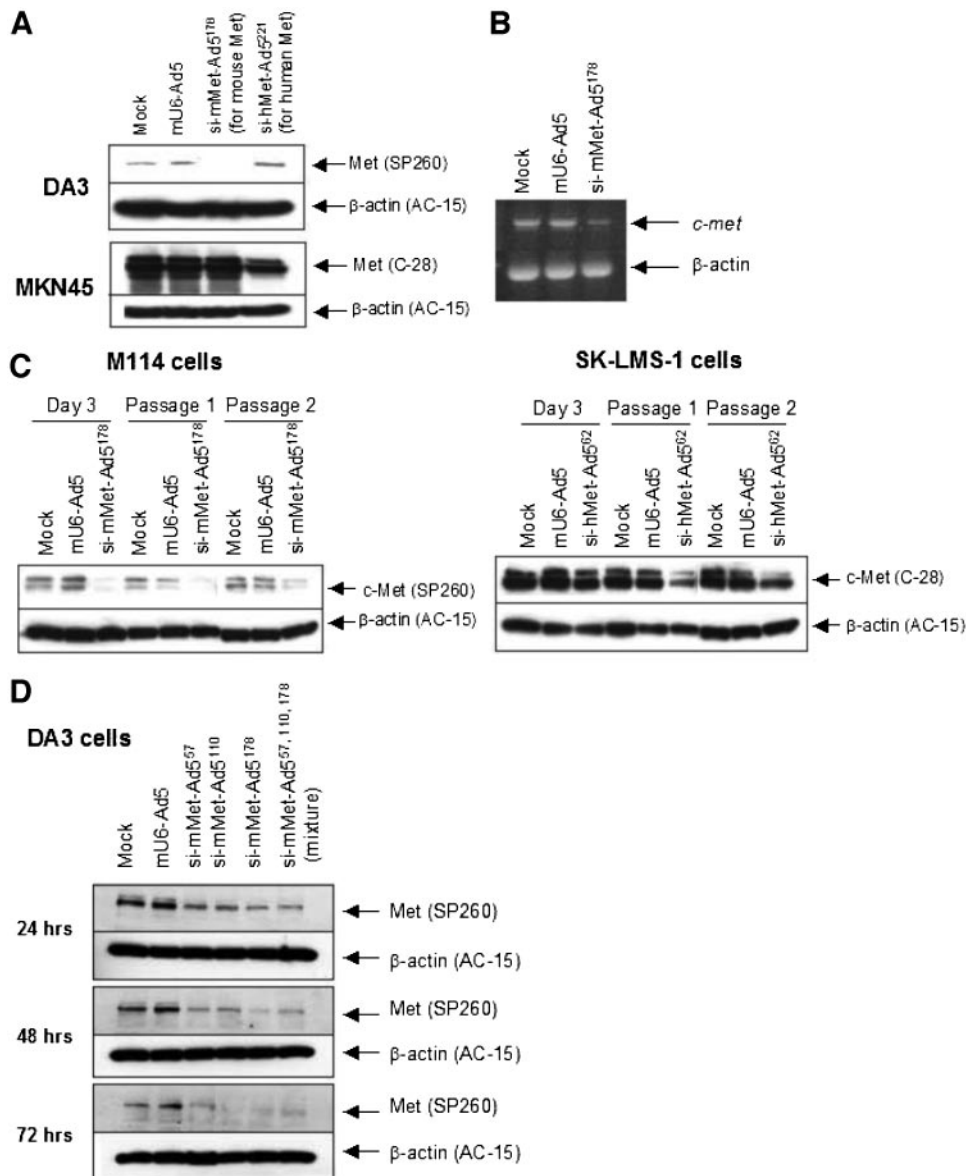


Fig. 2. Specificity and time course of Met RNAi. A, specificity of the Ad Met siRNA. DA3 mouse mammary adenocarcinoma cells and MKN45 human gastric cancer cells were infected with control virus and si-mMet or si-hMet-Ad5 viruses at m.o.i. 100. In DA3 cells, Met expression (day 3) was strongly suppressed only in the cells infected with si-mMet-Ad5<sup>178</sup>; no reduction in Met protein was observed in cells infected with the most potent RNAi for human Met, si-hMet-Ad5<sup>221</sup>. In MKN45 cells, Met expression was suppressed only in the cells infected with si-hMet-Ad5<sup>221</sup>. B, analysis of *met* mRNA expression by reverse transcription-PCR. Total RNA was extracted from the DA3 cells infected with control mU6-Ad5 or si-mMet-Ad5<sup>178</sup> viruses at m.o.i. 100 for 3 days. Expression of *met* mRNA was dramatically reduced only in si-mMet-Ad5<sup>178</sup>-infected cells. C, RNAi persistence after cell passage. Left: M114 cells were infected with si-mMet-Ad5<sup>178</sup> at m.o.i. 100. Three days after infection, cells were trypsinized and reseeded (Passage 1). This was repeated after 3 more days (Passage 2). RNAi persisted even through passage 2. Right: SK-LMS-1 cells were infected with si-hMet-Ad5<sup>62</sup> at m.o.i. 100, and Met expression was followed up to the second passage as described for M114 cells. No remarkable reduction in Met expression was seen 3 days after infection, but significant suppression was observed after passage 1 and through passage 2. D, time course of Met reduction by *c-met* siRNA adenovirus. DA3 cells were infected with si-mMet-Ad5 viruses at m.o.i. 100. Cells were harvested after 24, 48, or 72 hours after infection, and Met expression level was determined by Western blot. Thirty micrograms of protein was loaded in each lane. The RNAi effect was observed with all of the si-mMet-Ad5 viruses from 24 hours to 72 hours. Adenovirus si-mMet-Ad5<sup>178</sup> produced the strongest effect. A mixture, of all three (si-mMet-Ad5<sup>57</sup>, <sup>110</sup>, <sup>178</sup>) was no better. This RNAi effect lasted after cell passage, but a reduction of cell viability was observed with si-mMet-Ad5-infected cells (data not shown).



in a similar manner. SK-LMS-1 cells are refractory to si-hMet-Ad5 infection compared with other human cell lines, and Met expression was not markedly affected at m.o.i. 100 on day 3 (data not shown). However, after cell passage Met expression was suppressed, suggesting that replating the cells facilitates adenovirus-mediated RNAi (Fig. 2C, right). Collectively, these results show that the RNAi effect can last through multiple cell divisions.

DA3 cells were infected with si-mMet-Ad5 viruses at m.o.i. 100, and Met expression was followed for 3 days (Fig. 2D). At 24 hours, the Met expression decreased in all of the infected cell groups but not in mock-infected or control virus (mU6-Ad5)-infected cells. At 48 and 72 hours, reduction in Met expression was more dramatic and, in all cases, Met expression was 25% or less relative to control cells. The si-mMet-Ad5<sup>178</sup> was most effective, and by 72 hours Met expression was nil. A mixture of the three, si-mMet-Ad5<sup>57</sup>, <sup>110</sup>, <sup>178</sup>, was no more effective than si-mMet-Ad5<sup>178</sup> alone. Similarly, we tested the Met expression in the DA3 cells after cell passage. RNAi lasted beyond one passage (data not shown). However, the RNAi effect was so dramatic that the cells could not be maintained, suggesting RNAi strongly interfered with cell viability. Because Met is a survival factor

(10), we next tested several cell lines for evidence of apoptosis after Met RNAi.

**Met RNAi Induces Apoptotic Cell Death.** We infected DA3 cells with si-mMet-Ad5<sup>178</sup> at m.o.i. 10, 50, and 100, and apoptotic changes were observed by TUNEL assay (Fig. 3A). Three days after infection, cell growth was suppressed in a dose-dependent fashion (data not shown), and some dark staining apoptotic cells were observed in si-mMet-Ad5<sup>178</sup>-infected cells. On day 6, the ratio of TUNEL-positive cells increased dramatically, and many apoptotic cells were observed at m.o.i. 100. Similar dramatic apoptotic changes were observed in MKN45 and PC-3 cells after infection with si-hMet-Ad5<sup>221</sup>, and virtually all of the cells with Met RNAi showed some degree of apoptosis (data not shown). By contrast, M114 cells did not show TUNEL-positive cells, as might be expected as the parental cells are NIH3T3 cells. M114 cells are autocrine for HGF/SF and Met (37). Suppression of Met expression and disruption of the autocrine loop morphologically changed the cells to a nontransformed phenotype (data not shown).

In general, the growth inhibition induced by Ad Met siRNA infection correlated with apoptosis. Interestingly, proliferation of the au-

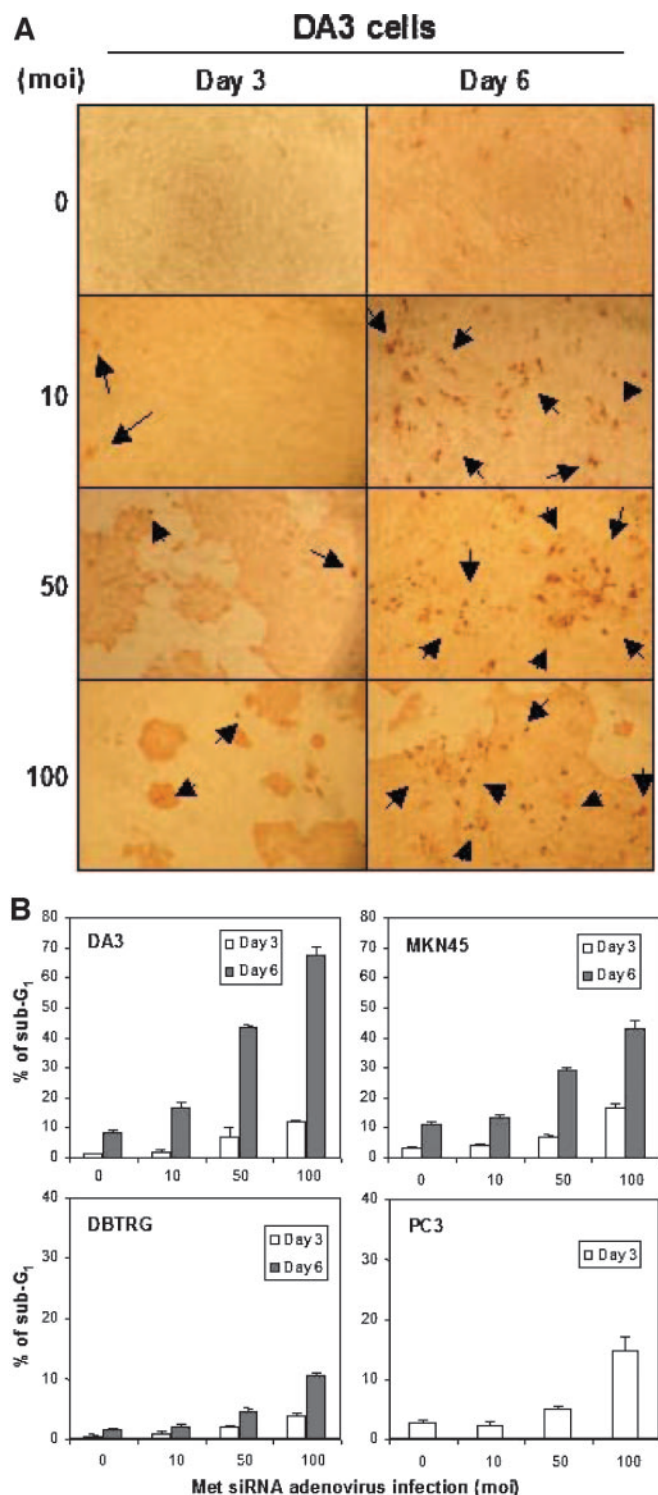


Fig. 3. Met RNAi induces apoptosis in tumor cells. **A**, TUNEL assay of si-mMet-Ad5<sup>178</sup> adenovirus-infected DA3 cells. The DA3 cells were infected with si-mMet-Ad5<sup>178</sup> viruses at different m.o.i. (0, 10, 50, and 100). Cells were fixed with formaldehyde at day 3 and 6, and apoptotic cells were detected by a TUNEL assay. Apoptotic cells are stained with dark brown (arrows). Three days after infection, suppression of cell growth is obvious at m.o.i. 50 to 100, and there are some apoptotic cells in si-mMet-Ad5<sup>178</sup> adenovirus-infected cells. At day 6, many apoptotic cells are detected at m.o.i. 10 to 100. **B**, Analysis of apoptosis in DA3, MKN45, DBTRG, and PC-3 cells after infection with Ad Met siRNA. Cells were infected with Ad Met siRNA (si-mMet-Ad5<sup>178</sup> for DA3 and si-hMet-Ad5<sup>221</sup> for MKN45, DBTRG, and PC-3) at m.o.i. of 10, 50, and 100. Three and 6 days after infection, both detached and adherent cells were collected and stained with phosphatidylinositol (Materials and Methods). The cells were processed by a flow cytometric analysis, and sub-G<sub>1</sub> fraction, the apoptotic fraction, was calculated. Three days after infection, the cells began to become apoptotic at m.o.i. 50 to 100. At day 6, dramatic increases in the apoptotic fraction were observed in DA3 and MKN45 cells. Bars,  $\pm$ SD.

tocrine SK-hepatocyte growth factor (HGF) cells was more strongly inhibited by Met RNAi than the parental SK-LMS-1 cells (data not shown). This suggests that proliferation of SK-HGF cells is more dependent on autocrine HGF/SF-Met signaling. Apoptosis in Ad Met siRNA-infected cells was additionally characterized by examining the sub-G<sub>1</sub> fraction (Fig. 3B). Cells were infected with Ad Met siRNA at m.o.i. of 10, 50, and 100 and examined by FACS analysis. DA3 and MKN45 cells were most susceptible to the loss of Met expression. The sub-G<sub>1</sub> fraction of DA3 cells 6 days after infection was 67% at m.o.i. 100. No loss of viability was observed in DA3 cells infected with control mU6-Ad5 virus (data not shown). Moreover, the sub-G<sub>1</sub> fraction of MKN45 cells was very high and at 43% by day 6. DBTRG cells also displayed an increase in apoptosis. Moreover, the sub-G<sub>1</sub> fraction of PC-3 cells at m.o.i. 100 examined after only 3 days was 15% and substantially higher than the control cells. Our results show that ligand-independent Met activity can be a requirement for tumor cell viability.

**Met RNAi on TR6LM Canine Prostate Carcinoma Cells and Nontransformed MDCK Cells.** We tested the effect of three different si-dMet-Ad5 viruses at m.o.i. 50 and 100 on nontransformed canine MDCK cells and TR6LM prostate carcinoma cells. All three viruses were very effective at knocking down Met expression in both MDCK and TR6LM cells (Fig. 4A). The growth and viability of TR6LM canine prostate cancer cells was adversely affected (data not shown), whereas the viability and proliferation of MDCK cells was not obviously affected. However, MDCK cell scattering was suppressed, and the scattering response paralleled the level of Met reduction (Fig. 4B), showing that reduction of Met expression can affect Met-dependent cell scattering without affecting cell viability or proliferation.

**Met RNAi Suppresses Ligand-Independent Met Signaling and Tumorigenesis.** On HGF/SF treatment, Met is phosphorylated and activates downstream pathways such as the phosphatidylinositol 3'-kinase-Akt (10, 11, 38) and the mitogen-activated protein kinase pathways (39). These pathways are essential for mediating biological activities such as cell migration, proliferation, morphogenesis, and survival. In DA3 cells endogenous phosphorylation of Met and p44/42 MAPK are observed in the absence of HGF/SF even after 24 hours serum starvation (Fig. 5A, top). This suggests that either other signaling pathways are active in these cells or ligand-independent Met activation is responsible for phosphorylation of Met and signals to MAPK (but not Akt). After HGF/SF treatment, DA3 cells show increased levels of Met phosphorylation for 2 hours, and then Met phosphorylation decreases (data not shown). The p44/42 MAPK and Akt are also rapidly phosphorylated (10 minutes after HGF/SF stimulation). Phosphorylation of p44/42 MAPK lasts longer in these cells.

In both mock-infected and control mU6-Ad5 virus-infected DA3 cells, Met was rapidly phosphorylated in response to HGF/SF (Fig. 5A, top). Increased phosphorylation of Akt and p44/42 MAPK was also observed after HGF/SF treatment. Although p44/42 MAPK phosphorylation was again observed in the si-mMet-Ad5<sup>178</sup>-infected cells, it was substantially suppressed compared with mock-infected or mU6-Ad5 control virus-infected cells. However, the phosphorylation of Met and Akt was almost completely abolished by si-mMet-Ad5<sup>178</sup> (Fig. 5A, top). Since Akt activation contributes to the stimulation of the antiapoptotic pathway, the reduced level of phospho-Akt may explain the increased susceptibility of si-mMet Ad5<sup>178</sup>-infected DA3 cells to apoptotic cell death. Alternatively, constitutive MAPK activation may contribute to ligand-independent survival in these cells.

MKN45 cells show strong phosphorylation of Met and its downstream molecules, p44/42 MAPK, even under serum-starved conditions. The phosphorylation status did not change even after treatment with HGF/SF, suggesting that the phosphorylation of Met in this cell

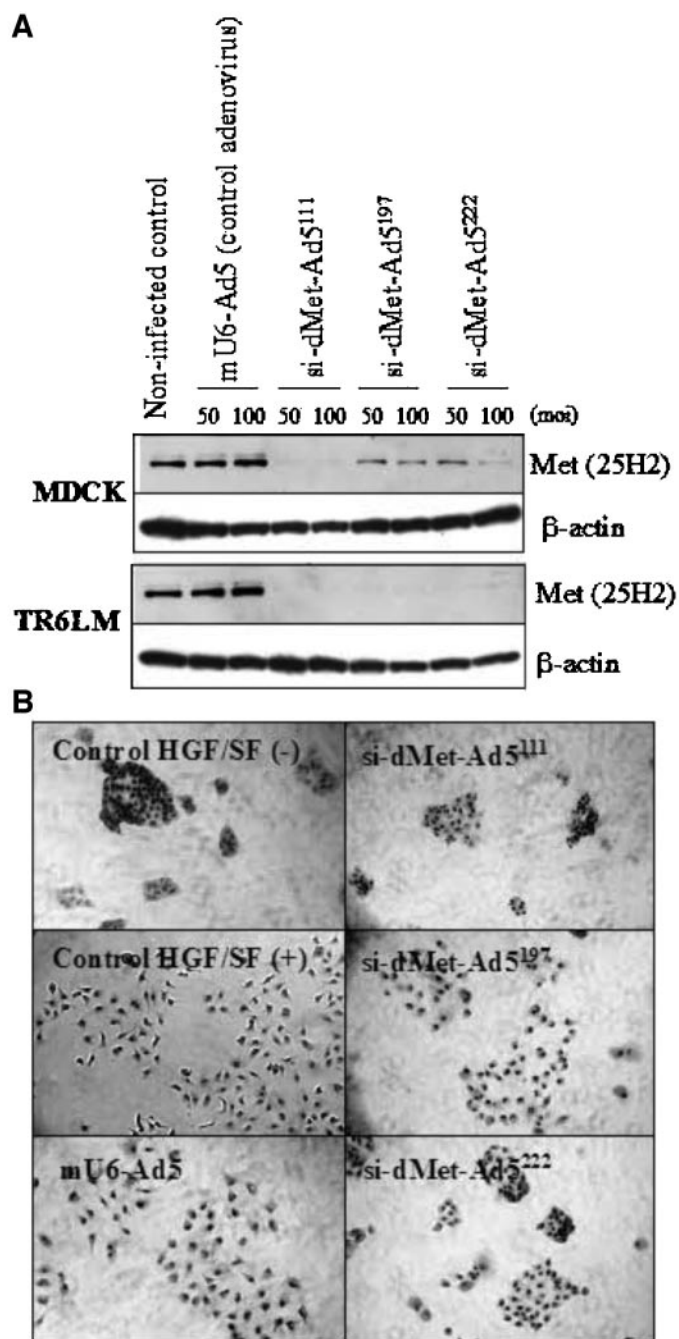


Fig. 4. RNAi on MDCK nontransformed canine epithelial cells versus TR6LM canine prostate carcinoma cells. **A**, MDCK and TR6LM cells were infected with three different constructs of Ad Met siRNA at different m.o.i. (50 and 100). After 3 days, cells were harvested, and Met expression level was observed by Western blot. Thirty micrograms of protein was loaded in each lane. The order of Met suppression in MDCK was si-dMet-Ad5<sup>111</sup> > si-dMet-Ad5<sup>222</sup> > si-dMet-Ad5<sup>197</sup>. In TR6LM cells, Met expression was almost completely inhibited by each of these three Ad Met siRNA constructs. **B**, RNAi on scattering of MDCK cells. MDCK cells were infected with mU6-Ad5 or si-dMet-Ad5 number 111, 197, or 222 at m.o.i. 50 and cultured for 3 days. After reseeding, cells were cultured for 24 hours and treated with 25 ng/mL HGF/SF for 24 hours. Cell scattering activity was suppressed in a Met-dependent manner (100× magnification). Thus, si-dMet-Ad5<sup>197</sup>-infected MDCK scattered the most and expressed the highest level of Met, whereas the si-dMet-Ad5<sup>111</sup>-infected cells scattered the least and expressed the lowest level of Met.

line is ligand-independent and, similar to the situation in DA3 cells, substantially signals through MAPK. After infection with si-hMet-Ad5<sup>221</sup>, Met phosphorylation was dramatically suppressed, and phosphorylation of both Akt and p44/42 MAPK disappeared completely after 24 hours serum starvation (Fig. 5A, bottom). The si-hMet-

Ad5<sup>221</sup>-infected cells partially responded to HGF/SF with p44/42 MAPK phosphorylation, but Akt phosphorylation was totally suppressed. Thus, DA3, MKN45, and PC-3 (data not shown) all display Met phosphorylation in a ligand-independent fashion (Fig. 5A), which appears to be required for the viability of these cells.

We tested whether si-Met-Ad5 RNAi would also suppress tumorigenicity. First, we asked whether DA3 cells infected with si-mMet-

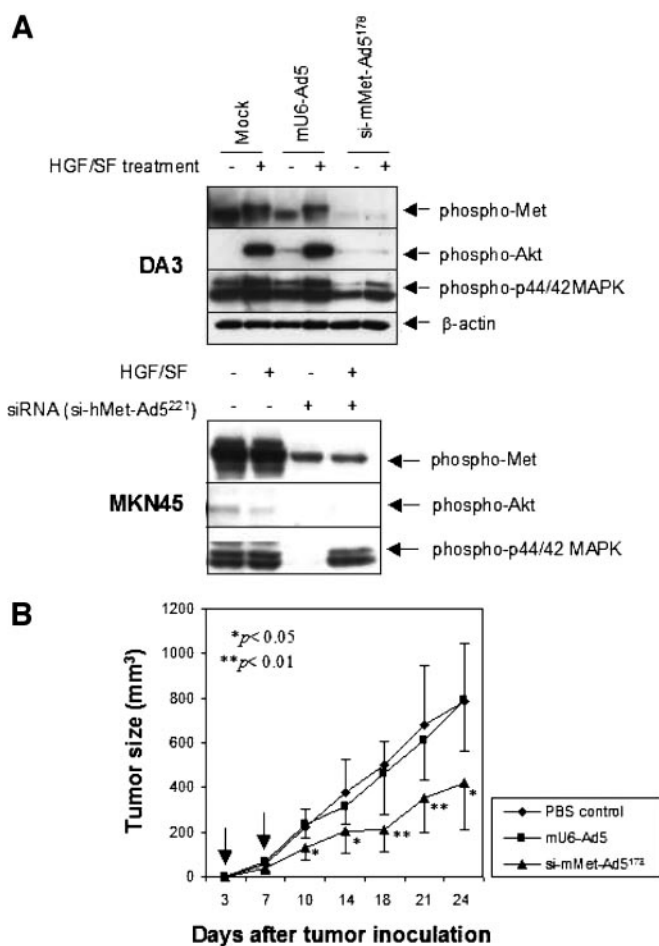


Fig. 5. RNAi suppresses Met signaling and tumor growth *in vivo*. **A**, Phosphorylation of Met and downstream signaling after Ad Met siRNA infection. *Top*: DA3 cells were infected with si-mMet-Ad5<sup>178</sup> virus for 3 days. Cells were transferred to serum-free medium and cultured for 24 hours. Cell lysates were collected 10 minutes after HGF/SF (100 ng/mL) treatment, and protein phosphorylation was analyzed by Western blot. After HGF/SF treatment, mock-infected and control virus-infected cells showed phosphorylation of Met, Akt, and MAPK. Phosphorylation of these molecules was marginal or inhibited in si-mMet-Ad5<sup>178</sup> virus-infected cells. There was no obvious change in the expression level of nonphosphorylated Akt in si-mMet-Ad5<sup>178</sup>-infected cells (data not shown). *Bottom*: MKN45 cells were infected with si-hMet-Ad5<sup>221</sup> virus for 2 days. Cells were transferred to serum-free medium and cultured for 24 hours. Cell lysates were collected 10 minutes after HGF/SF (100 ng/mL) treatment, and protein phosphorylation was analyzed by Western blot. Equal amount of protein (30 μg) was loaded in each lane. Mock-infected control cells show strong Met phosphorylation and Akt and p44/42 MAPK activation even in the absence of serum, and HGF/SF treatment did not show any remarkable changes in phosphorylation. Cells infected with si-hMet-Ad5<sup>221</sup> show dramatic reduction in Met phosphorylation, and there was no phosphorylation of Akt and p44/42 MAPK in the serum-starved cells. After HGF/SF treatment cells show p44/42 MAPK phosphorylation but no Akt phosphorylation. **B**, Met RNAi on *in vivo* tumor growth. DA3 cells (1 × 10<sup>5</sup>) were inoculated subcutaneously in the right flank of BALB/c mice. Three and 7 days after tumor inoculation, 4 × 10<sup>7</sup> pfu of si-mMet-Ad5<sup>178</sup> virus (in 0.1 mL PBS), 4 × 10<sup>7</sup> pfu of mU6-Ad5 virus (in 0.1 ml PBS), or 0.1 ml PBS only was injected into the tumor (arrows), and effect of the treatment was observed. Tumor size was followed and recorded for 24 days. PBS control: 0.1 mL PBS only, mU6-Ad5: 4 × 10<sup>7</sup> pfu in 0.1 mL PBS. Each symbol represents eight to ten animals; bars, ±SD. \*, *P* < 0.05 compared with PBS control and *P* < 0.01 compared with control (mU6-Ad5) virus treatment. \*\*, *P* < 0.01 compared with both PBS control and mU6-Ad5 virus treatment. There was no statistically significant difference between PBS control and mU6-Ad5 virus-treated groups. (HGF, hepatocyte growth factor; SF, scatter factor)

Ad5<sup>178</sup> *in vitro* would grow as tumors subcutaneously in BALB/c mice. Tumor growth was measured for 4 weeks, and there was a dramatic difference. When DA3 uninfected or control mU6-Ad5 virus-infected cells were inoculated simultaneously, all mice (10 of 10) showed remarkable tumor growth over a 4 week period. By contrast, tumor formation by DA3 cells infected with si-mMet-Ad5<sup>178</sup> was almost completely suppressed, and a tumor grew in only 1 of 11 mice inoculated (data not shown). Presumably, there was no *in vivo* rescue from the RNAi-induced apoptosis.

We also asked whether si-mMet-Ad5 could suppress tumor formation *in vivo*. DA3 cells were inoculated subcutaneously in the flank of BALB/c mice. On days 3 and 7 after tumor inoculation, si-mMet-Ad5<sup>178</sup> was injected directly into the tumor. Mice injected with si-mMet-Ad5<sup>178</sup> virus showed a significant reduction in the tumor size ( $P < 0.05$  to  $0.01$ ), whereas there was no difference in tumor size between PBS control and mU6-Ad5 virus-treated mice (Fig. 5B). This suggests that Met RNAi has therapeutic potential.

## DISCUSSION

RNAi is a strong tool for silencing the function of specific genes (24–26). In this study, we produced siRNA adenoviruses carrying target sequences against either mouse, canine, or human Met. The different adenoviral constructs showed marked suppressive activities on Met protein expression (Fig. 1 and Fig. 4A), and the suppression of Met by these siRNA adenoviruses was quite reproducible. For instance, the order of Met suppression in mouse cell lines (M114 and DA3) was si-mMet-Ad5<sup>178</sup> > si-mMet-Ad5<sup>110</sup>, si-mMet-Ad5<sup>57</sup> > si-mMet-Ad5<sup>60</sup> (Fig. 1A and Fig. 2D). In human cell lines (DBTRG, and PC-3) the order was si-hMet-Ad5<sup>221</sup> > si-hMet-Ad5<sup>62</sup> > si-hMet-Ad5<sup>16</sup> (see Fig. 1, B and C). In addition, si-hMet-Ad5<sup>221</sup>, which worked the best in human cell lines, did not affect Met expression in mouse DA3 cells, whereas si-mMet-Ad5<sup>178</sup> produced a dramatic reduction in the Met protein level (Fig. 2A). This was confirmed at the mRNA level (Fig. 2B). And in MKN45 human cancer cells, Met expression was suppressed only by si-hMet-Ad5<sup>221</sup> but not by si-mMet-Ad5<sup>178</sup> (see Fig. 2A). These results indicate that our siRNA constructs produce RNAi in a sequence-specific manner.

We also tested the duration of RNAi with the siRNA adenovirus system. In a previous report, in dividing cells, silencing lasted only 3 to 7 days, presumably because of the dilution of siRNA with each cell division (40). In our experiments, however, the strongest effects were observed after the first cell passage (day 6 to 7 after infection), and RNAi was still evident after passage 2 (see Fig. 2C), indicating that RNAi persists at least 9 to 10 days after Ad Met siRNA infection. It is possible that our adenoviral constructs containing a U6 promoter-based RNA transcription system produce at high M.O.I. more siRNA molecules per cell compared with direct transfection of siRNA molecules. After si-mMet-Ad5 infection, RNAi was induced swiftly, and ~50% reduction in Met expression was observed by 24 hours (Fig. 2D). The effect of RNAi continued to increase for up to 72 hours.

The efficiency of Met reduction by si-Met-Ad5 varied among the cell lines. DBTRG, PC-3, and MKN45 cells (see Fig. 1, B–D) responded better than SK-LMS-1 (Fig. 2C), and the suppression of Met expression in DA3 cells was more robust than in M114 cells (Fig. 1A and Fig. 2D). Entry of adenovirus requires two receptors: a primary receptor, Coxsackie and adenovirus receptor for attachment, and secondary receptors such as the  $\alpha_v\beta_3$  and  $\alpha_v\beta_5$  integrins for internalization (41). Recently, lack of Coxsackie and adenovirus receptor expression was reported to be one of the major limiting factors to adenovirus gene therapy (42). Because the cell lines of epithelial origin (PC-3, MKN45, and DA3) are expected to express higher levels of receptors to adenovirus than nonepithelial lines (SK-LMS-1 and

M114), the sensitivity to infection might explain the differences in Met reduction, rather than other factors such as transcription efficiency of siRNA molecules after internalization. However, with SK-LMS-1 cells, cell passage enhanced the RNAi effect dramatically (Fig. 2B).

Abrogation of HGF/SF-Met signaling to suppress Met-dependent tumorigenesis has been achieved by several different approaches. For example, a dominant-negative form of Met has been reported to reduce *in vitro* motility and invasiveness, as well as the *in vivo* tumorigenic and metastatic potential of DA3 cells (19). In the context of Met signaling, however, direct molecular targeting against the Met protein would be a more straightforward and robust way to prove its biological contribution. Abounader *et al.* (21, 22) designed a U1snRNA/ribozyme for targeting Met and reported that it reversed the malignancy of glioma cells, inhibited the growth and angiogenesis, and promoted apoptosis. In these experiments, gene expression was inhibited in terms of both the mRNA and protein levels at 73 to 98% by stable expression of U1snRNA/ribozyme. However, the efficiency with transient infection with an adenovirus system remained at 75% reduction in mRNA and 50% reduction in Met protein. Our Ad Met siRNA system produced efficient reduction in Met as revealed by Western blot analysis. Reduction in Met expression reached 62% in DBTRG cells, 68% in PC-3 cells, and 71% in MKN45 cells (Fig. 1, B–D). In the more sensitive DA3 cell line, the reduction was 85 to 100% (Fig. 2, A and D). Although there is a variation in the susceptibility to Ad Met siRNA among the cell lines, this RNAi system provides a powerful method for achieving reduction in Met expression.

Perhaps the most important finding is that RNAi by Ad Met siRNA induces cell death in many of the tumor cells we have tested *in vitro* and can possibly do the same *in vivo*. Although the level of Met expression did not necessarily correlate with the susceptibility to apoptosis, a reduction in Met protein triggered extraordinary cell death in carcinoma cell lines displaying ligand-independent Met activation such as DA3 and MKN45 and in other cell lines as well (Fig. 5A). By contrast, M114 cells after Ad Met siRNA infection showed morphologic changes, but the virus did not trigger apoptosis (data not shown). We expect this is because of the original nontransformed NIH3T3 phenotype of an immortal cell line. With Met siRNA adenovirus infection of MDCK cells, Met expression and cell scattering was effectively inhibited (Fig. 4, A and B), but the growth and viability of these nontransformed cells were unaffected (data not shown). In contrast, TR6LM tumor cells infected with si-dMet-Ad5 viruses showed remarkable suppression of Met expression, cell growth, and apoptotic changes (Fig. 4A; data not shown). Normal and malignant epithelial cells differ in their response to HGF/SF (43), and the canine Met siRNA adenovirus data suggests that like PC-3, MKN45, DA3 cells Met is crucial for tumor cell survival but perhaps not for normal cells. This is consistent with a recent study with a conditional Met liver knockout (44). In these animals the liver is normal until challenged for survival and tissue remodeling, in which case the liver is impaired. Liver-specific Met conditional knockout mice survive without obvious liver damage, suggesting that Met knockdown in normal tissue may not severely affect viability. Moreover, *in vivo* treatment of subcutaneously inoculated DA3 cells with Ad Met siRNA showed significant reduction in tumor growth (Fig. 5B), but no deleterious effects on the animal were obvious.

On HGF/SF stimulation, the Met receptor is phosphorylated, and this event is followed by the recruitment of a group of signaling molecules and/or adaptor proteins to its cytoplasmic domain and multiple docking site (1). This action leads to the activation of several different signaling cascades that form a unique network of several different types of responses, such as proliferation, migration, invasion,

angiogenesis, and metastasis (1, 3, 16). After infection with si-mMet-Ad5, DA3 cells showed remarkable suppression of scattering, invasion, and proliferation (data not shown); similar results were observed in the human cell lines (PC-3 and SK-HGF; data not shown). Suppression of the phosphorylation of Met and of downstream molecules (Akt and p44/42 MAPK) was observed in si-mMet-Ad5-infected DA3 cells but not in mock-infected cells (see Fig. 5A). This suggests that all of the observed phenotypic changes in these cells were mediated by suppressing the phosphorylation of Met and downstream molecules. It has been known that HGF/SF signaling stimulates the Akt pathway and protects cancer cells from death (10, 11, 45, 46). HGF/Met is capable of protecting cells from apoptosis by both phosphatidylinositol 3'-kinase/Akt and MAPK pathways (10). In our study DA3 cells show very low Akt phosphorylation, but MAPK signaling was quite robust even in the absence of ligand (see Fig. 5A). Importantly, Met RNAi works on the cells that proliferate in a ligand-independent fashion (see Fig. 5A). Although the Akt pathway is quite important for viability, based on the Met RNAi-induced apoptosis, one is left to conclude that ligand-independent Met activation is required for survival, but this may not require phosphatidylinositol 3' kinase/Akt. In MKN45 cells, Met phosphorylation is quite robust after serum starvation, and the phosphorylation of Met and downstream MAPK are not additionally stimulated by HGF/SF treatment. However, after infection of MKN45 cells with si-hMet-Ad5 viruses, the phosphorylation of Met and p44/42 MAPK was strongly suppressed, as was cell growth (data not shown), and the cells undergo apoptosis (Fig. 3B). Presumably, ligand-independent Met activation is responsible for MAPK phosphorylation in both DA3 and MDCK cells. This suggests that the reduction of Met expression by RNAi can be an important strategy for suppression of tumor cell growth and survival.

Since overexpression or active mutation of the Met protein is involved in a wide spectrum of solid tumor cells, Met is considered a very important target molecule for cancer therapy (47, 48). Recently, *in vivo* therapies that target HGF/SF-Met signaling have been reported by several research groups. One approach is the use of the NK4-HGF/SF antagonist (49). However, this therapy may not be applicable to tumor cells in which Met signaling is ligand-independent. Other investigators have used the dominant-negative forms of Met (19, 20) or Met ribozyme (22, 50) as a targeting tool. These reports show substantial suppression of tumor growth. Here, we show that RNAi coupled with an adenovirus vector system represents an alternative therapy strategy that can effectively suppress Met expression in a sequence- and species-dependent fashion. Moreover, this approach can target a wide variety of tumor types and inhibit Met-dependent, malignant phenotypes *in vitro* and *in vivo*. Therefore, these viruses provide a powerful new tool to analyze the HGF/SF-Met signaling pathway and function as well as offer a new opportunity for cancer intervention.

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## RNA Interference Reveals that Ligand-Independent Met Activity Is Required for Tumor Cell Signaling and Survival

Nariyoshi Shinomiya, Chong Feng Gao, Qian Xie, et al.

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