A Hammerhead Ribozyme Suppresses Expression of Hepatocyte Growth Factor/Scatter Factor Receptor c-*MET* and Reduces Migration and Invasiveness of Breast Cancer Cells[1]

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ABSTRACT

Purpose: **Hepatocyte growth factor/scatter factor (HGF/SF), via its receptor c-MET, has been implicated to play a pivotal role in breast cancer development and progression. This study examined a transgene—consisting of a combination of U1snRNA, hammerhead ribozyme, and antisense, designed to inhibit c-*met* expression—and its impact on the migration and *in vitro* invasion of breast cancer cells.**

Experimental Design: **A hammerhead ribozyme targeting human c-*MET* was cloned into a modified pZeoU1EcoSpe vector and transfected into breast cancer cells MDA MB 231 and MCF-7 by electroporation. Expression of MET mRNA and protein was determined. Migration and *in vitro* invasiveness of transfected cells were also analyzed.**

Results: **Breast cancer cells were transfected with the ribozyme-containing plasmids. Stable transfectants manifested an almost complete loss of *MET* mRNA and protein, as shown by reverse transcription-PCR, Northern blotting, and Western blotting, respectively, whereas the wild-type plasmid had no effects. Met-ribozyme transfected cells exhibited reduced migration and *in vitro* invasiveness through extracellular matrix (Matrigel), compared with the wild-type cells and cells transfected with empty plasmid.**

Conclusions: **These data show that targeting c-*MET* by way of a hammerhead ribozyme encoding antisense to c-*MET* is an effective approach in reducing the invasiveness of breast cancer cells.**

INTRODUCTION

HGF,[3] also known as SF, is a cytokine that plays multiple roles in breast cancer (1, 2). *In vitro* studies have demonstrated the cytokine to be able to stimulate the dissociation, migration, motility, adhesion to, and invasion of extracellular matrix via mechanisms such as induction of the phosphorylation of focal adhesion kinase and paxillin (3–5). HGF/SF is also a potent angiogenic factor, both *in vitro* and *in vivo* (6–8). The action of HGF/SF is mediated by c-*MET*, a proto-oncogene, which has an intrinsic kinase domain (9, 10). Upon stimulation of HGF/SF, met becomes phosphorylated and initiates a range of signals that lead to activation of cellular behaviors.

Cancer cells, including breast cancer cells, overexpress HGF/SF receptor (3–5). Clinical studies have provided the most direct evidence that HGF/SF and its receptor are powerful factors associated with the progression and prognosis of patients with breast cancer (11–13). Breast cancer tissues express a higher level of MET, both at protein and mRNA levels. Breast tissues are rich in stromal cells, particularly fibroblasts that are the main producers of HGF/SF *in vivo*. Stromal fibroblasts from breast cancer tissues have been shown to express and produce large amounts of bioactive HGF/SF, compared with normal tissue stroma. The level of HGF/SF and MET are among the most relevant factors to predict the prognosis of patients bearing breast cancer (14, 15).

Given the clinical importance of HGF/SF and its receptor, to suppress the activity of HGF/SF will have strong clinical bearing. A growing number of agents and molecules are known to have an inhibitory effect on HGF/SF-induced action on cancer cells. These agents include invasion inhibiting factors (16), γ-linolenic acid (17, 18), IL-4, and IL-12 (19, 20) via mechanisms that indirectly associate with HGF/SF and its receptor. An antagonist specific to HGF/SF has also found to be able to suppress the action of HGF/SF by competing for the receptor with mature HGF/SF (21–23).

Recently, a powerful approach has been reported to target the receptor, c-*MET*, using U1/ribozyme, a hammerhead ribozyme the encodes a specific antisense sequence to c-*MET* (24). It was shown that the U1/ribozyme was able to reverse the malignancy of glioblastoma cells both *in vitro* and *in vivo*, thus offering a possible approach of gene therapy specifically targeting HGF/SF pathways. The current study examined whether the ribozyme was effective in manipulating the invasiveness of breast cancer cells.

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[3] The abbreviations used are: HGF, hepatocyte growth factor; SF, scatter factor; U1snRNA, U1 small nuclear RNA; RT-PCR, reverse transcription-PCR; IL, interleukin.

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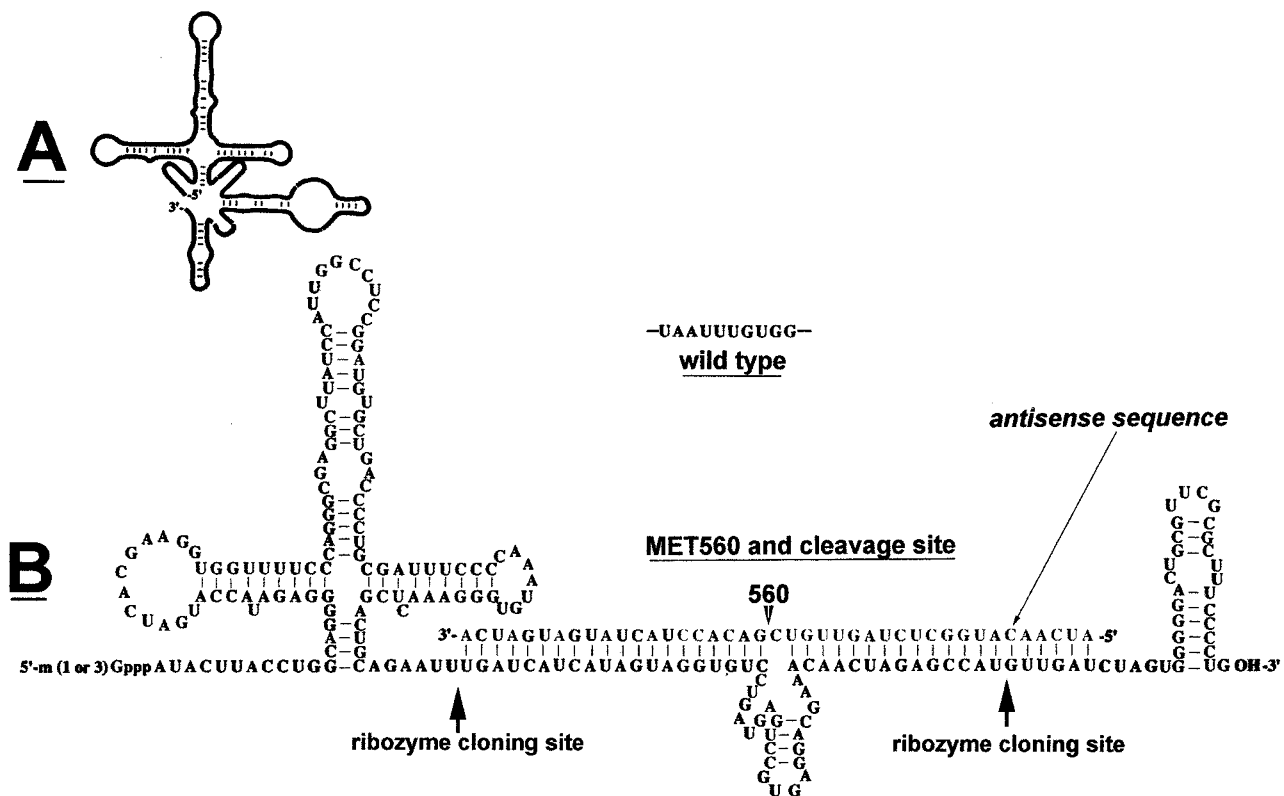

Fig. 1 Structure of ribozyme and U1snRNA. *A,* the secondary structure of U1snRNA. *B,* the structure of MET560 hammerhead and pU1. Antisense sequence is indicated. The hammerhead structure recognizes and cleaves the GUC position (MET560) at position 560, as indicated. In the control plasmid, a sequence as indicated replaces the antisense and hammerhead structure. *Arrows,* cloning sites for the ribozyme and control sequence.

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MATERIALS AND METHODS

Human breast cancer cells, MDA MB 231 and MCF-7, were from the European Collection of Animal Cell Culture (Salisbury, United Kingdom) and maintained routinely in DMEM with 10% FCS. Rabbit antibody to human c-MET (C28), mouse anti-β-actin, and chemiluminescence kit were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-conjugated antimouse and antirabbit antibodies were from Sigma Chemical Co. (Poole, Dorset, United Kingdom). Zeocin was from Invitrogen, Ltd. Recombinant human HGF/SF was a generous gift from Dr. T. Nakamura (Osaka, Japan). Matrigel (reconstituted basement membrane) was purchased from Collaborative Research Products (Bedford, MA). A transwell plate equipped with a porous insert (pore size, 8 μm) was from Becton Dickinson Labware (Oxford, United Kingdom).

U1/Ribozyme Targeting Human c-MET. This was constructed as reported previously (24, 25). Briefly, pZeoU1EcoSpe parent vector was derived from wild-type U1snRNA, which is an essential component of the spliceosome complex and is stable and abundant in the nucleus. The U1 promoter is cloned in a *Bam*HI site of a modified pZeo-EcoSpe vector, which is zeocin resistant. A hammerhead ribozyme that recognizes and cleaves the GUC sequence was used. The complementary pair of ribozyme oligonucleotides that span between 540 and 580 with a GUC sequence at position 560 of human c-MET was synthesized, annealed at 40°C, and ligated into pU1 at the *Eco*RI and *Spe*I site to create MET560 vector and referred to as MET560 in the text (Fig. 1). An irrelevant sequence was also cloned into pU1 between *Eco*RI and *Spe*I sites as control empty vector and is referred to as pU1 in the text (Fig. 1). Vectors were transfected into chemically competent *Escherichia coli*. Plasmid was extracted using a plasmid extraction kit (Qiafilter; Qiagen).

Electroporation and Establishing Stable Transfectants. Thirty μg of MET560 or PU1 wild-type plasmid were mixed with 8×10^6 cells for 2 min and then electroporated at 290 V and 1500 with an electroporator (Easyjet; Flowgen). Cells were immediately transferred to complete medium prewarmed to 37°C and plated into 25-cm^2 tissue culture flasks. After 24 h, selection began with zeocin at 100 μg/ml, and medium was changed every 3–4 days. After 4 weeks, cells were changed to maintenance medium (with 25 μg/ml G418). The existence of the plasmid was confirmed using PCR for the following primers: UBAMHF, 5′-ggatccgccaaccgaaagt-3′; and UBAMHR, 5′-gtacgattaacaactaaga-3′. Cells were subsequently frozen for later use.

Extraction of RNA, RT-PCR, and Northern Blotting. Cellular RNA was extracted using an RNA extraction kit (AbGene, Ltd., London, United Kingdom) and quantified using a spectrophotometer (Wolf Laboratories). cDNA was synthesized using a first-strand synthesis with an oligo dt primer (AbGene). The PCR primers used were as follows: for c-*MET*, 5′-GTC

CAG GCA GTG CAG CAT GTA-′3 and 5′ACT ATA GTA TTC TTT ATC ATA CAT GTC′3. The PCR was performed using a Perkin-Elmer thermocycler and PCR mastermix reaction mixture (Abgene, Surrey, United Kingdom): 5 min at 95°C and then 30 s at 94°C-60 s at 64°C (60°C for c-*MET*), 60 s at 72°C for 36 cycles, and finally 72°C for 7 min. For hammerhead structure, primers used were UBAMHF and UBAMHR. β-*actin* was amplified simultaneously using primers 5′-gctgatttgatg-gagttgga-3′ and 5′-tcagctacttgttcttgagtgaa-3′. PCR products were then separated on a 0.8% agarose gel, visualized under UV light, photographed using a Unisave camera (Wolf Laboratories), and documented with Photoshop software.

For Northern analysis, 10 μg of total RNA were resolved on a 0.8% denaturing agarose gel and transferred to a nylon membrane. A cDNA probe for human c-*MET* was used for subsequent hybridization overnight at 45°C in the presence of formamide. Membranes were washed under stringent conditions and then exposed to X-ray film. All blots were subsequently reprobed with a human β-*actin* cDNA to correct for loading errors. mRNA band densities of both c-*MET* and β-*actin* were determined using a volume analysis function of Molecular Analyst (Bio-Rad). c-*MET* levels are shown here as the ratio c-*MET*:β-*actin*.

Western Blotting Analysis of MET. Cells were lysed in HCMF buffer containing 1% Triton, 0.1% SDS, 2 mM $CaCl_2$, 100 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 1 μg/ml aprotinin for 30 min before clarification at 13,000 × *g* for 10 min. Protein concentrations were measured using fluorescamine (Sigma Chemical Co.) at 200 μg/ml and quantified by using a multifluoroscanner (Denly, Sussex, United Kingdom). Equal amounts of protein from each cell sample (15 μg/lane) were added onto a 8% polyacrylamide gel. After electrophoresis, proteins were blotted onto nitrocellulose sheets and blocked in 10% skimmed milk for 60 min before probing with the antihuman MET or antihuman β-actin antibody and peroxidase-conjugated secondary antibodies. A molecular weight marker mixture (SDS-6H; Sigma Chemical Co.) was used to determine the protein size. Protein bands were visualized with a chemiluminescence system (Santa Cruz Biotechnology). Exposed films were scanned with a scanner, and the density of protein bands was analyzed with the software Optimas [Optimas (UK) Ltd., Milton Keynes, United Kingdom].

***In Vitro* Invasion Analysis.** This was done as previously reported and modified in our laboratory (25, 26, 27). Briefly, transwell inserts with 8-μm pore size were coated with 50 μg of Matrigel and dried, before being rehydrated. Cells (20,000) were added to each well with or without HGF/SF. After 96 h, cells that had migrated through the matrix and stuck to the other side of the insert were fixed, stained with 0.5% (w/v) crystal violet, and counted under microscope.

Cell Migration Motion Analysis and Colony Dissociation Assay. Cells were plated out at 10,000 and 20,000/well for colony scattering assay (MCF-7 cells) and for motion analysis (23, 28, 29). For cell scattering, MCF-7 cells only were tested as they formed tight colonies, whereas MDA MB 231 grew in monolayers and were not suitable for the test. Cells were allowed to adhere for an additional 24 h to form colonies. Medium or HGF/SF (40 ng/ml) was then added. After an additional 24 h, cells were fixed and stained with 0.5% w/v crystal violet and either photographed or analyzed as reported previously (28). For cell motion analysis, cells were first overlaid with light mineral oil and then placed on a stage heated to 37°C. Culture medium or HGF/SF (to the final concentration at 40 ng/ml) was added to the medium under the mineral oil. Cells were then subjected to continuous monitoring using a digital camera (Panasonic Digital) and time lapse video recorder. Images were subsequently obtained at 10-min intervals and analyzed using a motion analysis package (Optimas 6). The accumulated distance that a cell traveled and the average speed over a period of 10 min were analyzed. Over 20 cells were analyzed in each setting, and data were automatically processed with Excel.

Statistics. Statistical analysis was carried out using the Mann-Whitney *U* test, and a significant difference was taken at $P < 0.05$.

RESULTS

Introduction of Plasmid DNA into Breast Cancer Cells and Selection of Stable Transfectants. After electroporation, a majority of cells were recovered and allowed to grow in plain medium for an additional 24 h. Zeocin at a concentration of 100 μg/ml was added to the cells, and medium was changed every 3–4 days. Cells without plasmid began to die from the second

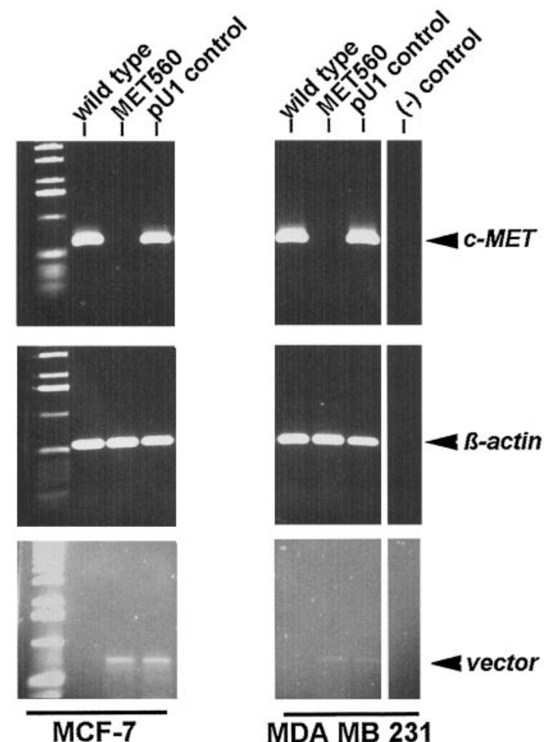

Fig. 2 Presence of hammerhead ribozyme plasmid in stable transfectants. After 4 weeks selection with zeocin, cells were pelleted, and RNA was extracted. RT-PCR was carried out with primers to detect c-*MET* (*top panels*), Hammerhead sequence (*middle panels*), and β-*actin* in MDA MB 231 cells (*left panels*) and MCF-7 cells (*right panels*). Cells carrying MET560 almost completed inhibited c-*MET* mRNA, whereas control plasmid had no effects on the expression.

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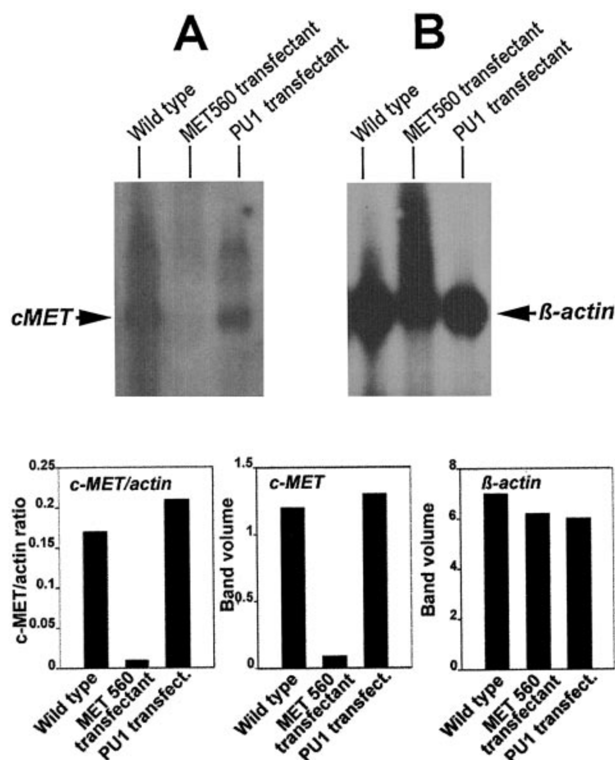

Fig. 3 Northern blotting for c-*MET* mRNA in MDA MB 231 cells. *Top panel: A,* membrane probed with specific human c-*MET* cDNA; *B,* membrane probed with β-*actin* cDNA. *Bottom panel, right,* band volume of β-*actin*, quantified by Molecular Analyst (Bio-Rad); *middle,* band volume of c-*MET; left,* volume ratio (c-*MET*:β-*actin).* MET560 greatly decreased the expression of c-*MET* mRNA.

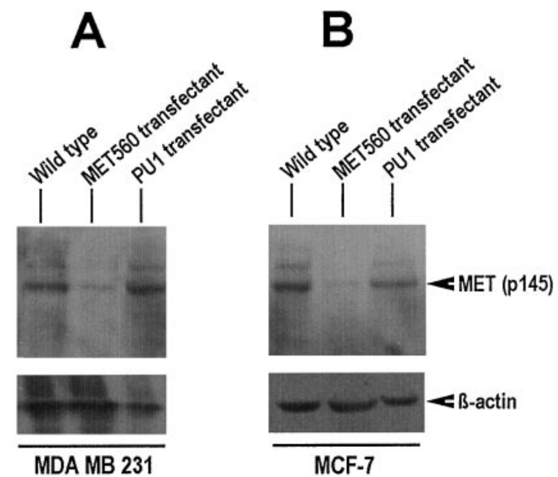

Fig. 4 Western blotting for Met in MDA MB 231 (*left*) and MCF-7 cells (*right*). MET was detected using a polyclonal antihuman MET antibody (rabbit, C28) and β-actin using a monoclonal antihuman β-actin antibody. MET560 reduced the level of MET in both cells, whereas the control plasmid (pU1) had no effects. The level of β-actin was not affected by either of the plasmids.

week of selection, and by the end of 4 weeks selection, cells were changed into maintenance medium (zeocin at 25 μg/ml).

The presence of plasmid DNA was verified using RT-PCR with primers specific to the Hammerhead ribozyme (UBAMHF and UBAMHR). MET560 was seen in MDA MB 221 (MET-MDA MB 231) and MCF-7 (MET-MCF-7) cells (Fig. 2, *middle panel*). Wild-type PU1 were successfully introduced into the cells and referred to as PU1-MDA MB 231 and PU1-MCF-7, respectively. In contrast, wild-type cells had no signal for the presence of plasmid.

c-*MET* mRNA Was Inhibited by U1/Ribozyme Carrying c-*MET* Antisense Sequence. Fig. 2 showed that in PU-1 and MET 560 transfected cells, the signal for the ribozyme was seen clearly (*middle panels*), as shown using RT-PCR with plasmid specific primers (UBAMHF and UBAMHR). Among the transfected cells, the MET560 transfected cells displayed a dramatic reduction of c-*MET* mRNA in both MDA MB 231 cells (*top left*) and MCF-7 cells (*top right*), when specific primers to human c-*MET* were used. This is in clear contrast to the wild-type and empty plasmid transfected cells, which showed the strong existence of c-*MET*. The reduction of c-*MET* mRNA was further confirmed by a Northern blotting analysis (Fig. 3), in which MET560 plasmid transfected cells displayed a reduction of c-*MET* mRNA, which is also reflected by a reduced ratio of c-*MET*:β-*actin.* This was in clear contrast to that of wild-type and PU1 transfectant, in which c-*MET* was strongly expressed.

The reduction of mRNA was reflected at protein level, as shown by Western blotting analysis (Fig. 4). Wild-type cells, particularly MDA MB 231 cells, demonstrated a high level of c-MET (Fig. 4, *left*). PU1 transfected cells exhibited a similar level of c-met. However, MET560 significantly reduced the MET protein level. A similar pattern was seen with MCF-7 cells (Fig. 4, *right*).

Reduction of *in Vitro* Migration in MET560 Transfected Cells. A motion analysis showed that there were changes of migration in MDA MB 231 cells (Fig. 5) and MCF-7 cells (Fig. 6) after being transfected with U1/ribozyme. In wild-type cells, stimulation with HGF/SF resulted in a rapid migration both at the migration distance (*top left* in Fig. 5 for MDA MB 231 cells) and migration speed (*bottom left* in Fig. 5 for MDA MB 231 cells). Interestingly, cells reached high migration speed 10 min after stimulation, as we reported previously on other cells (28). Cells carrying MET560 plasmid lost their response to HGF/SF (*middle panel* in Fig. 5 for MDA MB 231), when compared with the wild-type cells (*left panel* in Fig. 5). Furthermore, MET560 transfected cells exhibited a lower migration speed (*middle bottom* of Fig. 5), when compared with the wild type (*left bottom* of Fig. 5). This is likely to be the result of the endogenous HGF/SF produced by breast cancer cells and its possible HGF/SF-MET autocrine effects, as demonstrated in our previous study (28). A similar response was seen with MCF-7 cells (Fig. 6). Control vector-transfected cells exhibited a similar pattern of migration to that of wild-type cells (*left* and *right panels* of Figs. 5 and 6), 10 min after HGF/SF stimulation. However, the early burst (within 10 min) of migration speed in control vector cells (*right panel*) was lower than that of control cells. We do not have an explanation for this phenomenon, although the nonspecific effect of the vector cannot be ruled out.

This was further seen in MCF-7 cells in colony scattering

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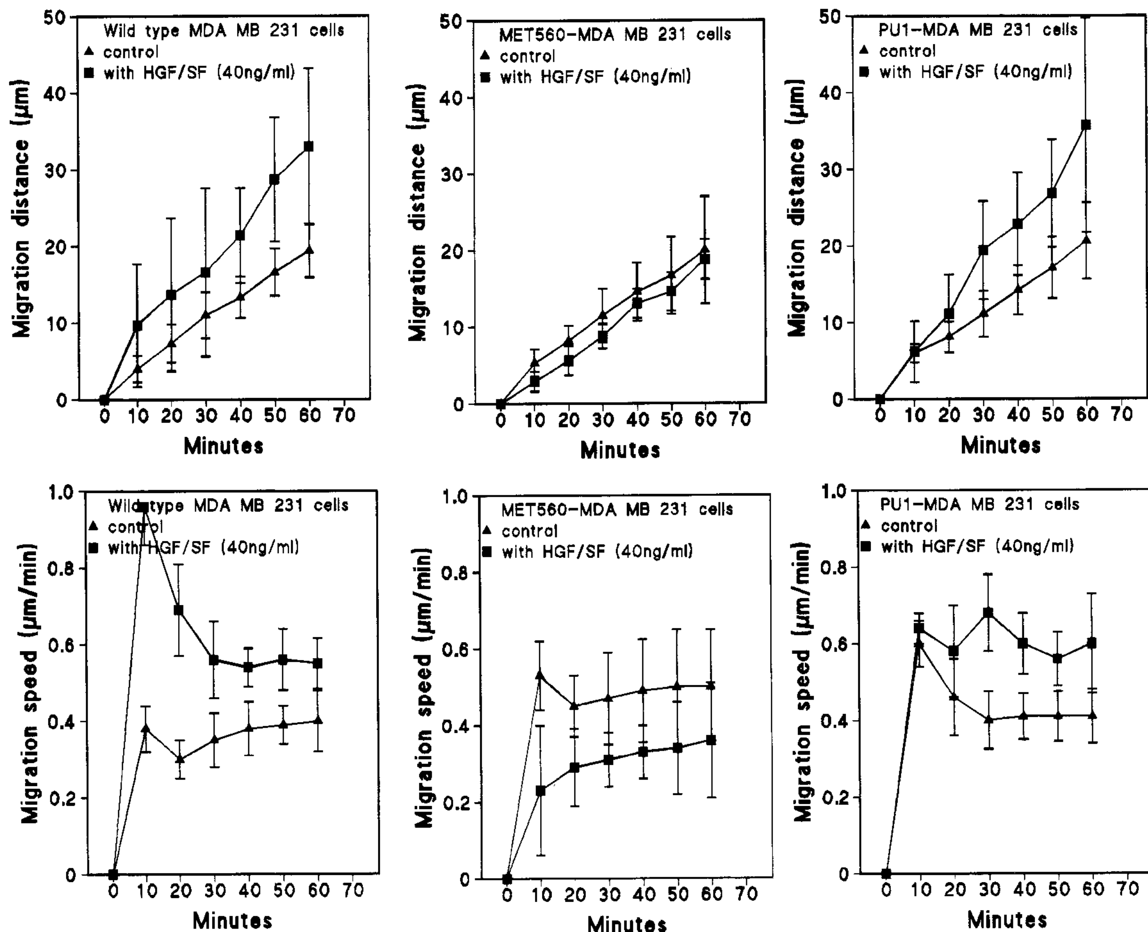

Fig. 5 Migration of MDA MB 231 cells. Wild-type (*left*), MET560 transfected (*middle*), and pU1 control plasmid (*right*) transfected MDA MB 231 cells were plated in a 24-well plate and allowed to adhere for overnight. They were then overlaid with light mineral oil and either HGF/SF or medium (control) was added. Cells were then subject to continuous time-lapse video recording on a heated stage (37°C). Series digitized images were analyzed using a motion analysis function in Optimas 6. The accumulated migration distance (µm; *top panel*) and average migration speed (µm/min; *bottom*) are shown here. HGF/SF increased the migration in both wild-type and pU1 transfected cells but not in the MET560 transfectant. *Bars,* SE.

(dissociation) assay. HGF/SF induced an increased dissociation (increase in the size of colonies), which was lost after transfection with MET 560 plasmid (Fig. 7).

Reduction of Invasiveness. The impact of antisense ribozyme on cell invasiveness was evaluated with an *in vitro* invasion model using Matrigel. HGF/SF has been known to increase the *in vitro* invasiveness, as also demonstrated here (Fig. 8). MET560 transfected cells clearly lost response to HGF/SF.

DISCUSSION

This study has demonstrated that the migration and invasiveness of breast cancer cells, in response to HGF/SF, can be manipulated by using a U1/ribozyme targeting the expression of human c-*MET*, HGF/SF receptor. Hammerhead ribozyme is a special RNA structure that has enzymatic activity and cuts mRNA at special recognition sites, such as the GUC used in the current study (24, 25). The approach has been tested successfully over a few other molecules in both *in vitro* and *in vivo* studies (30–33). The ribozyme can be delivered as plasmid or as viral vectors, such as an adenovirus-based delivery system (34, 35). In this study, a GUC site at the human c-*MET* position 560 was chosen. This allows cleavage of c-*MET* mRNA at a specific site. Our study has shown that the pU1-MET560 transfected cells lost a vast majority of c-*MET* RNA, as revealed by RT-PCR and Northern blotting. This reduction of mRNA has been clearly reflected at the protein level. In contrast, a wild-type pU1 plasmid had no effects on the level of MET both at the mRNA and protein levels. This suggests that a hammerhead ribozyme encoding a specific antisense sequence is indeed a powerful approach to target a specific molecule, particularly tumor- and metastasis-related molecules, such as c-MET.

HGF/SF is a known invasion and migration stimulus to cancer cells (36, 37). This was shown clearly in the current study, in which wild-type cells became highly motile after stimulation with HGF/SF, exhibiting faster migration and longer traveling distance, and acquired invasiveness after stimulation. In the case of MCF-7 cells, which grow in colonies, they increased their scattering in response to HGF/SF. However, in cells that were transfected with MET560 plasmid and removed

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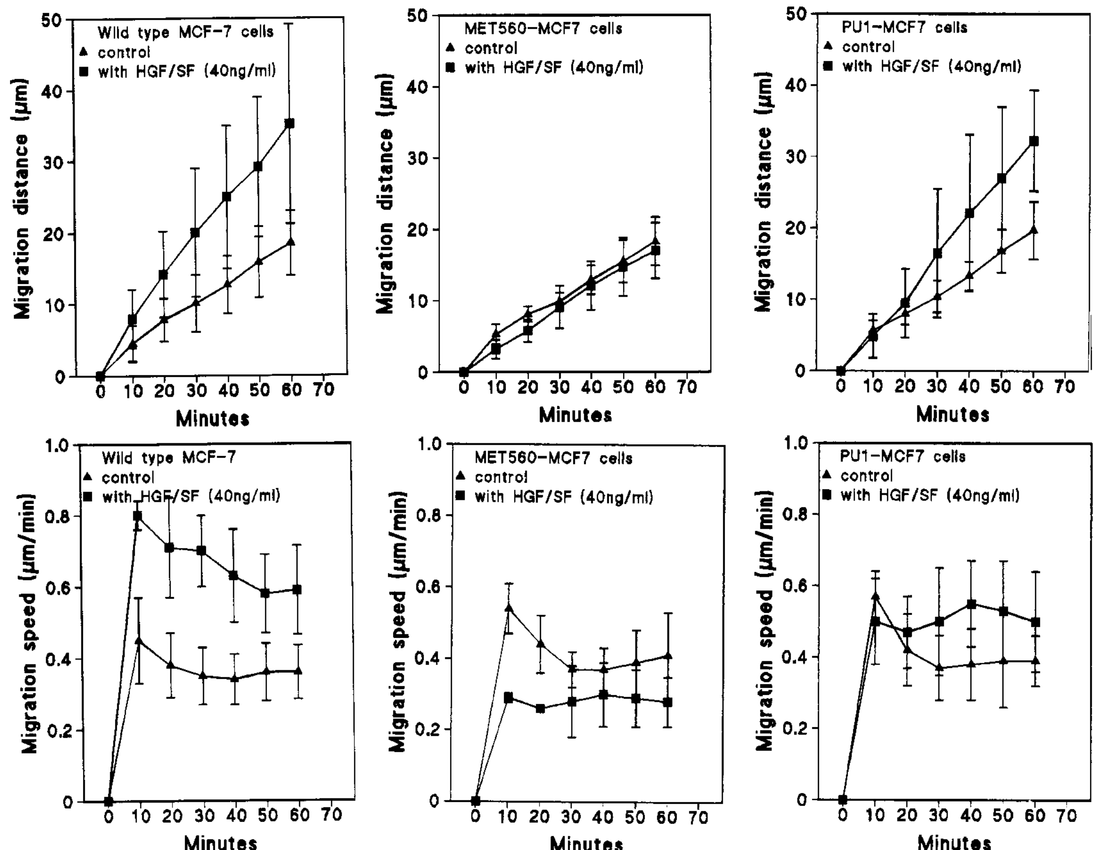

Fig. 6 Migration of MCF-7 cells. The setting is similar to that mentioned in Fig. 5. HGF/SF increased the migration in both wild-type and pU1 transfected cells but not in the MET560 transfectant. *Bars,* SE.

of their c-*MET*, they almost completely lost their response to HGF/SF. This not only indicates that HGF/SF is a powerful motility and invasion promoter in breast cancer cells, it also points to the possibility that targeting the HGF/SF receptor, c-MET, is a powerful approach in combating the action of HGF/SF in breast cancer.

Breast cancer is a tumor type that has high levels of c-MET in tumor cells and a high level of HGF/SF associated with the tumor (10–13, 36). In various studies, the majority of cancer cells in breast tumor overexpress c-MET, at both protein and mRNA levels. Furthermore, HGF/SF has been found to be expressed at a high level in breast tissues. The HGF/SF found in breast cancer tissues has been demonstrated to be bioactive. The source of HGF/SF in this case is also worth noting. Breast tissue, particularly breast tumor tissues, is known to be stroma rich. Histology easily reveals that the stromal cells in these tissues are mainly fibroblasts. It is the fibroblasts that contribute mainly to the high level of bioactive HGF/SF in breast cancer tissues. It has been sparsely reported that some breast cancer cells may also express and produce bioactive HGF/SF. However, the latter is a minor part of the contribution to the high level. The importance of HGF/SF and c-MET in breast cancer is particularly reflected in its correlation with the prognosis of patients. In a large scale study, HGF/SF and c-MET levels are found to be closely related with the prognosis of patients with

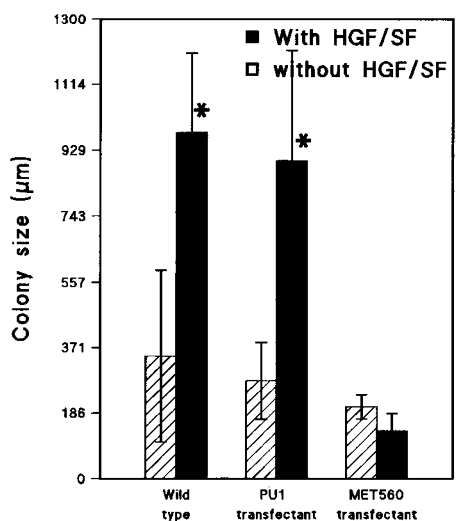

Fig. 7 Reduction of colony scattering of MCF-7 cells. MCF-7 cells were plated in a 24-well plate and allowed to form colonies. HGF/SF or medium was then added for an additional 24 h. Colonies were captured as digitized images, and sizes were analyzed using area morphometry. HGF/SF increased the size of colonies in both wild-type and pU1 transfected cells. However, MET560 transfected cells lost their response to the stimulus. *Bars,* SE.

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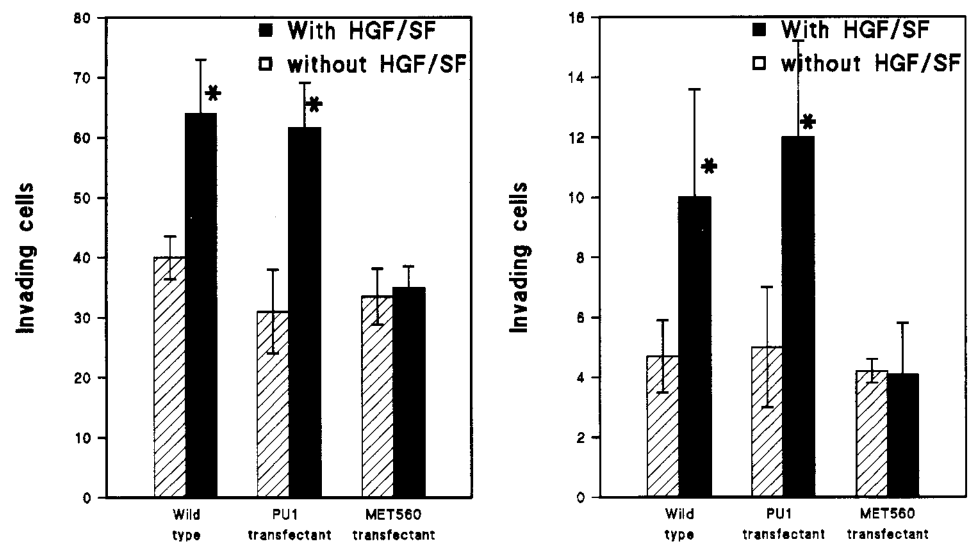

Fig. 8 *In vitro* invasion of MDA MB 231 (*left*) and MCF-7 (*right*). Matrigel (50 μg/insert)-coated inserts were added with cancer cells, together with HGF/SF or medium for 96 h. The number of invaded cells was given as number/high power field. *, $P < 0.05$ *versus* without HGF/SF. HGF/SF increased invasiveness in wild-type and pU1 transfected cells but not in MET560 transfected cells. *Bars,* SE.

breast cancer and perhaps the most powerful independent prognostic factors.

Strategies to counteract the action of HGF/SF have been long sought. The known approaches include using specific antagonist to HGF/SF, such as NK4, specific inhibitors to MET, and inhibitors that exert inhibition to HGF/SF in a nonspecific fashion such as γ-linolenic acid, IL-12, retinoic acid, and IL-4. The approach reported here may provide another important method to specifically target c-*MET* at the mRNA level. It also provides an opportunity to target the molecule for long-term gene therapy. Indeed, it has been reported that the approach has yielded promising results in glioblastoma in animal studies, in which the U1/ribozyme targeting c-*MET* mRNA reverses the malignant nature of neuroblastoma (24). Taken together, U1/ribozyme is a powerful tool to eliminate human c-*MET* from cancer cells. Targeted cancer cells exhibit a reduction of migration and invasion in response to HGF/SF, as demonstrated in the current study and other studies (24). Such an approach would be interesting in targeting tumor cells *in vivo*. However, conventional bacterial plasmids, such as the one used in the current study, offer very limited space in direct *in vivo* studies because of the low capacity of the bacterial plasmids to enter cells. We are currently developing a viral form of these ribozymes, aiming at direct delivery of these ribozymes *in vivo*.

In summary, this study reports that U1/ribozyme designed to target human c-*MET* is a powerful approach to inhibit the action of HGF/SF in human breast cancer cells. It specifically reduced the expression of c-*MET* at the mRNA and subsequently protein levels, and cells carrying the plasmid exhibited low invasive and low migratory properties in response to HGF/SF. It is further indicated that the ribozyme may be a powerful approach in suppressing the sinister action of HGF/SF in breast cancer.

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