

# Hepatocyte Growth Factor/c-Met Signaling Promotes the Progression of Experimental Human Neuroblastomas

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## ABSTRACT

Neuroblastoma is the most frequent solid childhood malignancy. Despite aggressive therapy, mortality is high due to rapid tumor progression to advanced stages. The molecules and mechanisms underlying poor prognosis are not well understood. Here, we report that cultured human neuroblastoma cells express the hepatocyte growth factor (HGF) and its receptor c-Met. Binding of HGF to c-Met triggers receptor autophosphorylation, indicating functional relevance of this interaction. HGF activates several downstream effectors of c-Met such as the mitogen-activated protein kinases extracellular signal-regulated kinase 1/extracellular signal-regulated kinase 2 and phospholipase C- $\gamma$ , whereas signal transducer and activator of transcription 3 is constitutively activated in neuroblastoma cells expressing c-Met. In addition, HGF is able to stimulate expression and proteolytic activity of matrix metalloproteinase-2 and tissue-type plasminogen activator in neuroblastoma cells, thereby promoting degradation of extracellular matrix components. We show that HGF stimulates invasion of neuroblastoma cells *in vitro* and *in vivo*, and it promotes the formation of angiogenic neuroblastomas *in vivo*. These processes can be blocked by specific inhibitors of the mitogen-activated protein kinase cascade, by inhibitors of phospholipase C- $\gamma$ , and also by the expression of a dominant negative signal transducer and activator of transcription 3 mutant. Our data provide the first evidence that the HGF/c-Met pathway is essential for invasiveness and malignant progression of human neuroblastomas. They further suggest that specific inhibitors of this pathway may be suitable as therapeutic agents to improve clinical outcome of neuroblastomas.

## INTRODUCTION

Neuroblastomas result from the malignant transformation of immature neural crest-derived cells. They represent the most frequent solid childhood malignancies and have a heterogenous biological and clinical behavior, ranging from complete regression without therapy to rapid progression despite intensive therapy. Several molecular abnormalities are known to contribute to the malignant progression of human neuroblastomas. These include amplification of the *MYCN* oncogene, chromosome 1p deletion, loss of CD44, TrkA, p75, and N-Ras (1–3). Although biologically and clinically relevant, these abnormalities do only partially account for the progression of human neuroblastomas. We hypothesized that hepatocyte growth factor (HGF) and its receptor c-Met might be determinants of neuroblastoma progression.

HGF, also known as “scatter factor,” was independently discovered as a growth factor for hepatocytes and stimulator of epithelial cell dissociation (4, 5). HGF is a multifunctional molecule produced by cells of mesenchymal origin. The mature, active HGF is a heterodimer composed of disulfide-linked  $\alpha$ - and  $\beta$ -chains (6). HGF binds with high affinity to its specific receptor c-Met, which is almost exclusively present on the surface of epithelial and endothelial cells (7–9). c-Met is synthesized as

a precursor protein of  $M_r$  170,000, further glycosylated, and subsequently cleaved into a  $M_r$  50,000  $\alpha$ -chain and a  $M_r$  140,000  $\beta$ -chain, as it matures on the cell surface (10–12). Binding of HGF to the extracellular domain of c-Met triggers phosphorylation of tyrosine residues within the kinase domain and subsequently of two COOH-terminally clustered tyrosine residues, which constitute the so-called multifunctional docking site. This site recruits downstream signaling molecules and adaptor proteins to activated c-Met, including phosphatidylinositol 3-kinase, c-Src, phospholipase C- $\gamma$ , Grb2, signal transducer and activator of transcription 3 (STAT3), and the SH2-domain-containing inositol phosphatase SHIP-1 (13–17). In parallel, HGF binding to c-Met activates matrix metalloproteinases (MMPs) and urokinase plasminogen activator (uPA; refs. 18 and 19). uPA and tissue-type plasminogen activator (tPA) convert enzymatically inactive plasminogen into active plasmin. MMPs and plasmin degrade proteins of the extracellular matrix, thus facilitating tumor cell invasion and metastasis.

The HGF/c-Met signaling pathway is essential for normal embryonic development, tissue regeneration, wound healing, and angiogenesis (reviewed in ref. 20). Thereby, HGF acts in a paracrine manner, and the pathway is transiently activated. The fine tuning of the HGF/c-Met signaling is disrupted in several tumors, resulting in a constitutive activation of c-Met. For example, c-Met is overexpressed in thyroid, ovarian, and pancreatic carcinomas, and overexpression is associated with metastatic spread and poor clinical outcome (21, 22). In addition, HGF and c-Met are often coexpressed in cells derived from solid malignancies (23, 24), setting the stage for an autocrine HGF/c-Met loop. In patients with hereditary and sporadic papillary renal carcinoma, several missense mutations of c-Met have been identified, leading to constitutive activation of the receptor (25, 26). Activation of the HGF/c-Met signaling pathway, which is known to be required for invasion of epithelial cells, leads to subsequent activation of the mitogen-activated protein kinase (MAPK) cascade, phospholipase C- $\gamma$ , Shp2, STAT3 (16, 27–30), and MMPs (19, 31). By triggering loss of cell–cell adhesion, changes of cell–matrix interactions, proteolytic breakdown of the extracellular matrix, enhanced cell migration, proliferation, and loss of apoptosis, HGF contributes essentially to tumor progression and metastasis.

Thus, the crucial role of HGF and c-Met in the progression of epithelial tissue-derived malignancies of the adult is now well established (reviewed in ref. 32). However, pediatric solid malignancies are almost exclusively derived from soft tissues but not from epithelia. Therefore, the role of the HGF/c-Met pathway in pediatric malignancies is less defined. In particular, it remains unclear whether this pathway is relevant for the progression of human neuroblastomas.

The present study was undertaken to elucidate whether c-Met and/or HGF is expressed in human neuroblastoma cells and whether their interaction contributes to neuroblastoma progression. A fundamental question was to investigate the mechanisms accounting for HGF-induced responses in neuroblastoma cells and whether such mechanisms could be blocked to provide novel approaches for an improved neuroblastoma therapy.

## MATERIALS AND METHODS

**Cell Culture.** Human neuroblastoma cell lines with various genotypes and phenotypes were cultured as described previously (33, 34). The cell lines

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SK-N-SH, SH-EP, and SH-SY5Y have normal *N-Myc* expression; and NMB, SMS-KAN, SMS-KCN, NGP, NLF, and KELLY harbor *N-Myc* amplification. Dishes and medium used for tissue culture were from Life Technologies, Inc. (Eggenstein, Germany). Human recombinant HGF was purchased from R&D Systems (Minneapolis, MN).

**Transfection of SH-EP Cells.** To obtain SH-EP neuroblastoma cells with dominant negative STAT3, the vector pCAGGS-Neo-hemagglutinin and the construct pCAGGS-Neo-hemagglutinin-STAT3F (35, 36) were transiently transfected into the cells with Superfect reagent (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Expression levels were monitored by Western blotting of equal amounts of whole-cell extracts with an anti-hemagglutinin antibody (Roche, Mannheim, Germany). The blots were subsequently stripped and reprobed with anti-phospho-STAT3 antibodies (Upstate Biotechnology Incorporated, Charlottesville, VA). Transfections were done in triplicate.

**Collection of Conditioned Medium.** To obtain supernatants for HGF determination, neuroblastoma cell cultures were washed three times with PBS and incubated for 24 hours with serum-free medium containing insulin (5  $\mu\text{g}/\text{mL}$ ), transferrin (5  $\text{mg}/\text{mL}$ ), and selenium (5  $\text{ng}/\text{mL}$ ; Sigma, St. Louis, MO). Conditioned medium was collected and centrifuged ( $2000 \times g$ , 15 min,  $4^\circ\text{C}$ ), and the supernatants were stored at  $-80^\circ\text{C}$  until further use.

**Hepatocyte Growth Factor Enzyme-Linked Immunosorbent Assay.** To determine HGF concentrations in conditioned medium, a HGF ELISA kit (R&D Systems) was used according to the manufacturer's protocol.

**Real Time Reverse Transcription-Polymerase Chain Reaction.** Relative quantification of mRNA expression for HGF, c-Met, MMP-1, MMP-2, MMP-9, uPA, tPA, and  $\beta$ -actin was done by real-time reverse transcription-PCR with Sybr Green Dye using a thermal cycler ABI PRISM 7700 (Perkin-Elmer-Applied Biosystems, Foster City, CA). Total RNA was prepared from  $10^6$  cells with an RNeasy kit from Qiagen according to the manufacturer's instructions. Only samples that gave an  $A_{260}/A_{280}$  ratio of 1.7 to 2.0 were used for additional applications. Reverse transcription-PCR was carried out as recommended (Invitrogen, Carlsbad, CA). cDNA was made from 1  $\mu\text{g}$  of total RNA, using random primers and Moloney murine leukemia virus reverse transcriptase. The RNA was primed for 10 minutes at  $60^\circ\text{C}$  and subsequently subjected to reverse transcription for 1 hour at  $37^\circ\text{C}$ , followed by heating at  $95^\circ\text{C}$  for 5 min. The PCR was performed with cDNA aliquots as template and the following primer sequences: HGF (forward), 5'-CCTAGATCTTTC-CAGTTAATCACACAAC-3'; HGF (reverse), 5'-TTCGGAGTCAGTGC-TAAAAAGAG-3'; c-Met (forward), 5'-TTAAGGAGACCTCACCATGTA-ATC-3'; c-Met (reverse), 5'-CCTGATCGAGAAACCACAACCT-3'; tPA (forward), 5'-GGCCTGTCTCCTTT CTATTTCG-3'; tPA (reverse), 5'-GCG-GCTGGATGGGTACAGT-3'; MMP-1 (forward), 5'-GGGAGATCATCGG-GACAACCTC-3'; MMP-1 (reverse), 5'-GGGCTGGTTGAAAAGCA-3'; MMP-2 (forward), 5'-TTCCTGGGCAACAAATATGAGA-3'; MMP-2 (reverse), 5'-TGGTCGCACCCACATCTTT-3'; MMP-9 (forward), 5'-GATC-CAAACTACTCGGAAGACTTG-3'; MMP-9 (reverse), 5'-GAAGGCG-CGGGCAA-3'; u-PA (forward), 5'-AAGGACAAGCCAGGCGTCTA-3'; u-PA (reverse), 5'-AAAATGACAACCAGCAAGAAAGC-3';  $\beta$ -actin (forward), 5'-GCATCCCCAAAGTTCACAA-3'; and  $\beta$ -actin (reverse), 5'-AGGA-CTGGGCCATTCTCCTT-3'. The primers were purchased from Thermohybrid (Ulm, Germany). PCR reactions were performed in triplicate using the PCR Mastermix for Sybr Green1-kit from Eurogentec (Seraing, Belgium) and the manufacturer's instructions. After an initial denaturation step of the cDNA (10 minutes at  $95^\circ\text{C}$ ), a two-step PCR was performed (15 s at  $95^\circ\text{C}$ ; 1 minutes at  $60^\circ\text{C}$ , 40 cycles). Template dilution experiments were performed to ensure that the efficiency of the PCRs for the target and the reference was approximately equal. Standard curves were calculated referring the threshold cycle ( $C_T$ ) to the logarithms of each cDNA dilution step. Detection of specific amplicons was assessed by generation of melting curves and agarose gel electrophoresis.  $C_T$  values obtained for HGF, c-Met, tPA, and MMP-2 were normalized by corresponding  $C_T$  values of  $\beta$ -actin. To generate the relative expression levels, each of the normalized HGF, c-Met, tPA, or MMP-2 values was divided by the normalized values of the calibrator. For HGF and c-Met quantification, we designated the neuroblastoma cell line SH-SY5Y as the calibrator. Quantification of tPA and MMP-2 expression was performed by comparing HGF-stimulated SH-EP cells with nonstimulated cells.

**Western Blot.** Twenty  $\mu\text{g}$  of clarified cell lysates were subjected to SDS-PAGE using 7.5% polyacrylamide gels. Gels were electrotransferred to nitro-

cellulose membranes (Amersham Biosciences, Piscataway, NJ). Immunodetection was carried out using appropriate antibodies. Anti-c-Met monoclonal antibody, polyclonal anti-phospho-c-Met antibody (Y1234/Y1235), polyclonal antibodies against phospholipase C- $\gamma$  and phospho-phospholipase C- $\gamma$ , STAT3, and phospho-STAT3 were from Upstate Biotechnology Incorporated. Polyclonal antibody against actin was from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-MAPK and anti-phospho-MAP-Kinase antibodies were obtained from Promega (Madison, WI). Antibodies against MMP-1, MMP-2, MMP-9, and uPA were from Calbiochem (San Diego, CA). tPA-specific antibodies were from Acris Antibodies (Hiddenhausen, Germany). Corresponding proteins were visualized by incubation with horseradish peroxidase-coupled antimouse IgG or antirabbit IgG (Dako, Hamburg, Germany) followed by detection with an enhanced chemiluminescence system (Amersham Biosciences).

**Zymography.** Cells were grown to approximately 70% confluence in 25  $\text{cm}^2$  flasks, washed three times with serum-free RPMI, and cultured with serum-free medium in the absence or presence of HGF (60  $\text{ng}/\text{mL}$ ) for 24 and 48 h. Supernatants were collected and centrifuged ( $2000 \times g$ , 20 min,  $4^\circ\text{C}$ ) to remove cellular debris. Conditioned media were concentrated with the Centricon 10 system (Amicon; Millipore, Bedford, MA). Each sample derived from  $5 \times 10^4$  cells was applied to SDS-PAGE on a 10% polyacrylamide gel with 0.1% (w/v) gelatin or 0.2% (w/v) casein. Gel electrophoresis was performed under nonreducing conditions without boiling. The gel was rinsed twice for 60 minutes in 2.5% (v/v) Triton X-100 to remove SDS and renature the proteins and incubated with activation buffer [50  $\text{mmol}/\text{L}$  Tris-HCl (pH 7.6) with 5  $\text{mmol}/\text{L}$   $\text{CaCl}_2$  and 0.02% (v/v)  $\text{NaN}_3$ ] overnight at  $37^\circ\text{C}$  with constant shaking. The gel was stained with 0.5% (w/v) Coomassie Brilliant Blue R-250 in 30% (v/v) methanol and 10% (v/v) acetate. Enzymatic activity was detected as a white band on the resulting blue background of undigested gelatin (casein).

**Cell Proliferation Assay.** Neuroblastoma cells were seeded into 96-well plates at a density of  $3 \times 10^3$  cells per well and cultured in 10% FCS containing RPMI medium in the absence or presence of HGF (100  $\text{ng}/\text{mL}$ ). Medium was replaced every 2nd day, and cultures were maintained for 7 days. The amount of viable cells was analyzed by measuring the conversion of the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide to formazan crystals. Tumor cells were incubated for 2 hours with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide solution (Gerbu Biotechnik, Gaiberg, Germany). Formazan crystals were solubilized, and the product was quantified spectrophotometrically by measuring the absorbance at 570 nm with a multiwell scanner.

**In vitro Cell Invasion Assay.** Assays were performed as described by Albini *et al.* (37). In brief, chambers with 8- $\mu\text{m}$ -pored polycarbonate membranes coated with Matrigel on the upper side were used (Becton-Dickinson, San Diego, CA). Cells ( $5 \times 10^5$ ) were seeded in FCS-free medium in the upper compartment, whereas medium supplemented with 10% FCS enriched with HGF (100  $\text{ng}/\text{mL}$ ) or without HGF was added to the lower compartment of each chamber. The following substances were tested: goat polyclonal neutralizing antibody against recombinant human HGF (40  $\mu\text{g}/\text{mL}$ ; Sigma); MEK/MAPK inhibitors PD98059 (30  $\mu\text{mol}/\text{L}$ ; Calbiochem) and U0126 (20  $\mu\text{mol}/\text{L}$ ; Promega); and phospholipase C- $\gamma$  inhibitor U73122 (2  $\mu\text{mol}/\text{L}$ ; Calbiochem). After 36 hours of incubation, cells invading the Matrigel and attaching to the lower surface of the membrane were stained with H&E and visualized microscopically at  $\times 200$  magnification. Cell counting was performed at  $\times 400$  magnification in 10 representative fields per well. All invasion assays were carried out in triplicate.

**Chorioallantoic Membrane Assay.** Fertilized eggs of the white Leghorn chick (*Gallus gallus domesticus*) were incubated at  $37.8^\circ\text{C}$  and 80% relative humidity. Cells of the human neuroblastoma cell line SH-EP were cultured to confluence in the presence or absence of HGF (60  $\text{ng}/\text{mL}$ ). Three days before harvesting, the MEK/MAPK inhibitors PD98059 (30  $\mu\text{mol}/\text{L}$ ) and U0126 (20  $\mu\text{mol}/\text{L}$ ) and the phospholipase C- $\gamma$  inhibitor U73122 (2  $\mu\text{mol}/\text{L}$ ) were added in separate experiments to the cell culture medium. Confluent cultures were harvested by trypsinization, washed once with medium, counted in a hemocytometer, and resuspended in cell culture medium to a final concentration of  $3 \times 10^6$  cells/50  $\mu\text{L}$  medium. The tumor cells were applied on the chorioallantoic membrane of 10-day-old chick embryos. For that purpose, a window was made into the eggshell of 3-day-old embryos and sealed with Durapore tape. The embryos were reincubated until day 10, when the tumor cells were added into Thermanox rings on the chorioallantoic membrane. Seven days after

inoculation, tumors were harvested and photographed under a dissection microscope. In separate experiments, tumors were treated between days 12 and 17 with the above-mentioned inhibitors (10  $\mu$ L of 0.5 mmol/L U0126, 10  $\mu$ L of 1 mmol/L PD98059, or 10  $\mu$ L of 0.1 mmol/L U73122 were added to each embryo every 2nd day).

**Immunofluorescence.** Tumors were fixed in 4% (w/v) paraformaldehyde and embedded in Tissue Freeze medium (Leica, Bensheim, Germany). Cryosections of 20  $\mu$ m thickness were fixed for 10 minutes in methanol. Nonspecific binding of antibodies was blocked by incubation with 1% (w/v) BSA for 10 min. The sections were incubated simultaneously with monoclonal antibodies against neurofilament (dilution 1:100; Dako) and smooth muscle actin (dilution 1:5000; Dako) for 1 h. After rinsing, the secondary Alexa Fluor<sup>®</sup>488-conjugated and Alexa Fluor<sup>®</sup>594-conjugated goat-antimouse antibodies (Molecular Probes, Eugene, OR; dilution 1:200) were applied for 1 h. After rinsing, slides were mounted under coverslips with fluorescent mounting medium (Dako), and the sections were studied with an epifluorescence microscope (Leica).

## RESULTS

**Cultured Neuroblastoma Cells Express Variable Amounts of Hepatocyte Growth Factor and c-Met.** We have examined a panel of human neuroblastoma cell lines for the expression of HGF and its receptor, c-Met. HGF was present at mRNA and protein levels in all investigated neuroblastoma cell lines. We found minute HGF expression in SMS-KAN, NMB, SH-EP, SK-N-SH, and NGP cells, whereas SMS-KCN, NLF, SH-SY5Y, and Kelly cells demonstrated considerable expression of HGF mRNA and protein (Fig. 1A and C). In addition, c-Met mRNA and protein were present at high levels in NMB, SH-EP, and SK-N-SH and at moderate levels in Kelly cells,

whereas c-Met was undetectable in SMS-KAN, SMS-KCN, NGP, NLF, and SH-SY5Y neuroblastoma cells (Fig. 1B and D).

**HGF Activates the Receptor c-Met in Human Neuroblastoma Cells.** To establish the functional competence of c-Met in human neuroblastoma cells, we explored the ability of HGF to trigger enzymatic activity of c-Met, *i.e.*, to induce rapid autophosphorylation of receptor tyrosine residues. In the absence of exogenously added HGF, c-Met of Kelly, SH-EP, and SK-N-SH cells displayed no basal tyrosine phosphorylation. In nonstimulated NMB cells, the receptor was found to be lightly phosphorylated (Fig. 2A). Exposure of NMB, SH-EP, and SK-N-SH neuroblastoma cells to exogenous HGF for the indicated periods (Fig. 2B) resulted in a strong tyrosine phosphorylation of c-Met, which occurred within the first 15 minutes after stimulation. The activation of the receptor was sustained for at least 2 h, as demonstrated by Western blot analysis using anti-phospho-Met antibodies.

**Activation of c-Met by Hepatocyte Growth Factor Stimulates Invasion, but not Proliferation, of Human Neuroblastoma Cells *In vitro*.** In an additional set of experiments, we investigated whether activation of the HGF/c-Met signaling pathway is related to invasion of NMB, SH-EP, and SK-N-SH neuroblastoma cells into Matrigel, a reconstituted basement membrane. In the absence of HGF, the cells were poorly invasive (Fig. 3A, 1–3). However, in the presence of HGF, the number of neuroblastoma cells able to invade the Matrigel increased 5- to 10-fold (Fig. 3A, 4–6, and B). This effect was completely blocked by addition of an HGF-specific neutralizing antibody, demonstrating that HGF is the key player of NMB, SH-EP, and SK-N-SH neuroblastoma cell invasiveness (Fig. 3A, 7–9). In

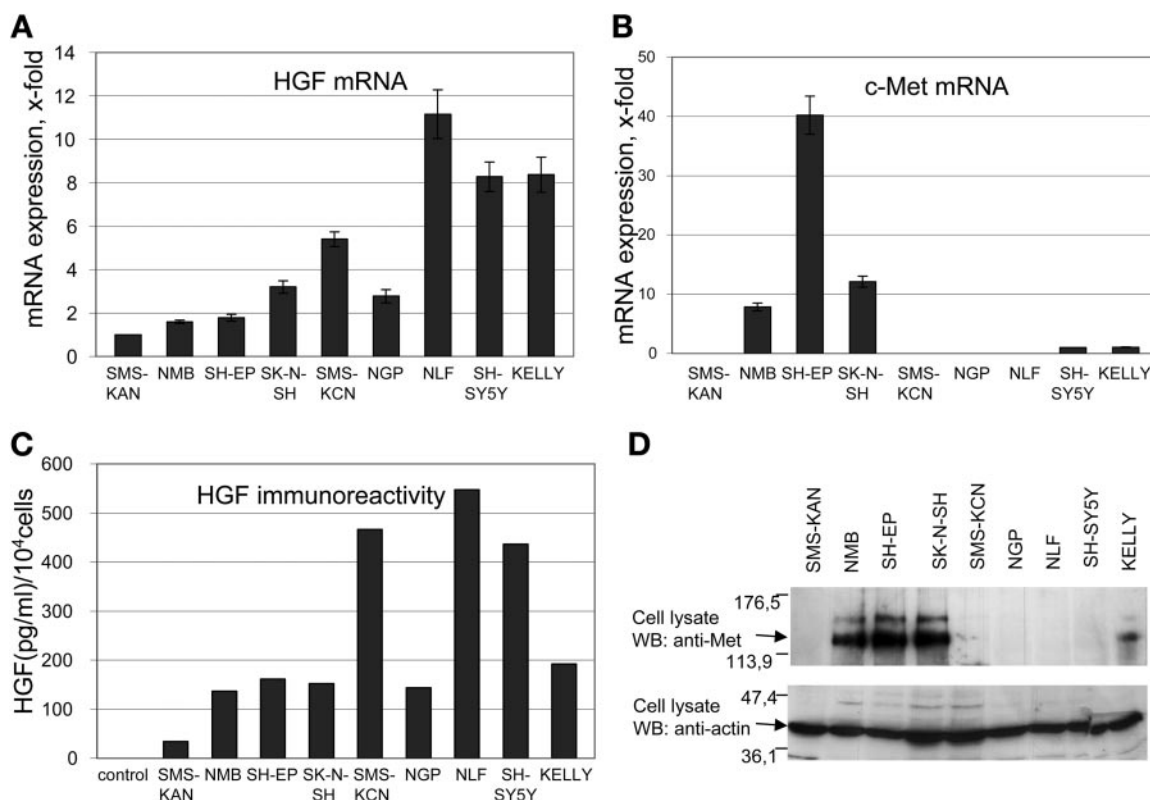


Fig. 1. Expression of HGF and its receptor c-Met in human neuroblastoma cell lines as determined by real-time reverse transcription-PCR analysis, ELISA, and Western immunoblots. A and B, real-time reverse transcription-PCR analysis of HGF/c-Met mRNA expression in neuroblastoma cell lines. The quantification was done as described in Materials and Methods; SH-SY5Y cells were designated as a calibrator. C, HGF immunoreactivity in supernatants of human neuroblastoma cells. The indicated cell lines were cultured with serum-free medium for 24 hours. HGF concentrations in the conditioned media were determined by HGF ELISA. Values are the means of triplicate determinations, which varied by less than 10% of the mean. D, expression of c-Met protein in neuroblastoma cell lines. Lysates from the indicated cell lines equalized for total protein were subjected to SDS-PAGE (20  $\mu$ g protein/lane) and Western blotting with antibodies against c-Met and actin, respectively. The faint higher band in the *top panel* represents the immature form of c-Met p170<sup>Met</sup>. The membrane was stripped and reprobbed with specific antibodies against actin to control equal loading.

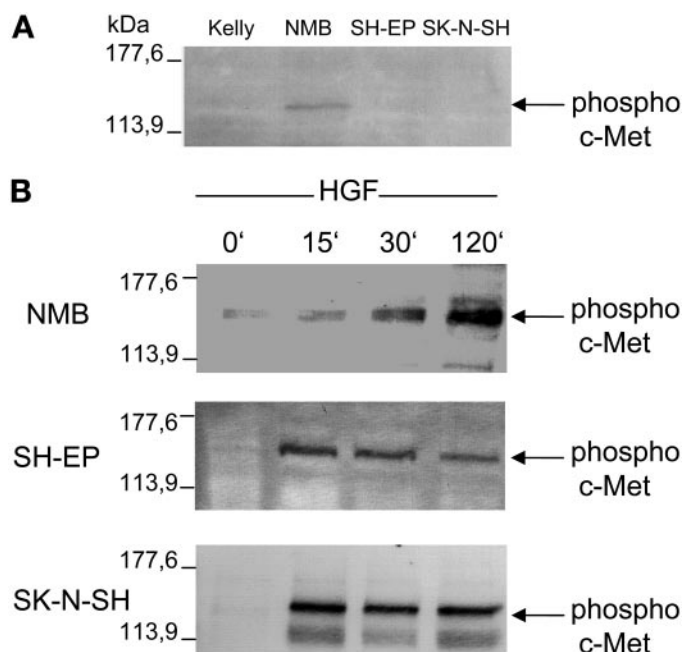


Fig. 2. c-Met protein autophosphorylation after treatment with HGF. The indicated human neuroblastoma cell lines were cultured in serum-containing medium (A) or serum-starved for 18 hours and subsequently treated with or without HGF (60 ng/mL) for 15, 30, and 120 minutes (B). Clarified lysates were equalized for total protein, subjected to SDS-PAGE (20  $\mu$ g/lane), and subsequently analyzed by Western blotting with phospho-Met specific antibodies (Y1234/1235).

contrast to the invasion-stimulating effects, HGF did not substantially alter the growth of NMB, SH-EP, and SK-N-SH neuroblastoma cells (Fig. 3C). These results indicate the biological relevance of the interaction between HGF and its receptor c-Met in human neuroblastoma cells.

**Hepatocyte Growth Factor Activates Multiple Signal Transduction Pathways in Neuroblastoma Cells.** We next sought to define the signaling molecules downstream of c-Met that are involved in HGF-mediated neuroblastoma invasiveness. We monitored the phosphorylation of several proteins and subsequently made use of specific inhibitors to investigate the roles of these proteins in mediating cell invasion into Matrigel.

We first studied whether the MAPK cascade is required for HGF-mediated invasiveness. A weak basal activation of the MAPK cascade was observed in SH-EP and SK-N-SH cells but not in NMB cells. HGF substantially stimulated phosphorylation of the ERK1/ERK2 (p44/p42) MAPKs in NMB, SH-EP, and SK-N-SH cells, as determined by immunoblot analysis of phospho-MAPKs relative to the absolute MAPK protein levels. Phosphorylation of MAPKs (p44/p42) occurred within 5 minutes after addition of HGF, and this effect persisted over a period of at least 2 hours (Fig. 4A). Exposure of the neuroblastoma cells to the selective MEK/MAPK inhibitors PD98059 or U0126 at concentrations as low as 30 or 20  $\mu$ mol/L, respectively, reduced their invasive potential to 15 or 27% of the HGF-stimulated control cells, indicating that the MAPK pathway is essential for neuroblastoma invasiveness *in vitro* (Figs. 5A, 4–5, and B).

HGF-mediated activation of phospholipase C- $\gamma$  is required for epithelial cell motility (28), but its potential contribution to neuroblastoma invasiveness has not yet been defined. As demonstrated in Fig. 4B, HGF induced phosphorylation of phospholipase C- $\gamma$  in neuroblastoma cells within 15 min, and phosphorylation was maintained for at least 2 hours (longer periods were not tested). The specific phospholipase C- $\gamma$  inhibitor U73122 suppressed HGF-mediated invasion of cells into Matrigel substantially, *i.e.*, to 36% of

controls at concentrations as low as 2  $\mu$ mol/L (Fig. 5A, 6, and B). This demonstrates that activation of phospholipase C- $\gamma$  is required for HGF-mediated invasiveness of human neuroblastoma cells *in vitro*. To ensure that the reduced invasiveness of neuroblastoma cells in the presence of inhibitors was not a secondary effect due to a slower proliferation, we treated the cells with the inhibitors mentioned above and studied cell growth. We observed that the concentrations used in the invasion assay had no effects on the proliferation rates over a period of 36 h, when the invasion assay was carried out (data not shown).

It has been reported, that STAT3 is critical for the c-Met-dependent formation of branched tubules, anchorage-independent growth, and tumorigenesis (16, 30, 38). We investigated whether, in addition to MAPKs and phospholipase C- $\gamma$ , STAT3 was involved in the HGF-mediated invasiveness of neuroblastoma cells. In fact, immunoblot analyses of phospho-STAT3 relative to total STAT3 levels revealed phosphorylation at tyrosine residues in c-Met-expressing neuroblastoma cells, which was not increased by exogenous HGF (Fig. 4C). These data suggest that STAT3 is constitutively activated and that this mechanism may contribute to neuroblastoma invasiveness. To validate this assumption, we transfected SH-EP neuroblastoma cells with a dominant negative STAT3 mutant carrying a phenylalanine substitution at tyrosine residue 705 (Y705F; refs. 35 and 36). Using Western blot analysis, we first confirmed inhibition of STAT3 in the transfectants (data not shown). We were then able to demonstrate that the transfectants had a markedly reduced ability to invade into Matrigel, even in the presence of HGF (Fig. 5A, 8). In contrast, control transfectants carrying the empty vector invaded the Matrigel as efficiently as the wild-type SH-EP cells, when stimulated with HGF (Fig. 5A, 7). These data demonstrate that constitutive activation of STAT3 is essential for the invasiveness of human neuroblastoma cells *in vitro*.

**HGF Stimulates Proteolytic Networks Involving MMP-2 and tPA in Human Neuroblastoma Cells.** The aggressiveness of tumor cells is dependent on their capability to degrade the extracellular matrix by activating certain proteases including plasminogen activator and MMPs (39). Using real-time reverse transcription-PCR, Western blotting, and zymographic analysis, we examined expression and activity of various matrix-degrading enzymes in neuroblastoma cells exposed to HGF. HGF stimulated expression and activity of MMP-2 and tPA (Fig. 6), but not uPA, MMP-1, or MMP-9 (not shown), in a time-dependent manner. Nonstimulated cells also showed MMP-2 and tPA expression on mRNA and protein levels but in lower amounts. The strongest expression for both proteases was observed 48 hours after stimulation with HGF (Fig. 6A and B). The zymographic analysis showed gelatinase activity of MMP-2 but not of MMP-1 and MMP-9. The band corresponding to the active form ( $M_r$  66,000) of MMP-2 disappeared in the presence of 20 mmol/L EDTA, confirming that the proteolytic activity originated from a member of the MMP family. In addition, zymographic analysis performed in casein containing gels for assaying uPA and tPA activation showed that the SH-EP cells produced proteolytically active tPA (band at  $M_r$  68,000), but not uPA (no bands at  $M_r$  54,000 and  $M_r$  33,000; Fig. 6C). These data demonstrate that HGF confers enhanced proteolytic potential to human neuroblastoma cells due to activation of tPA and MMP-2 and may thereby facilitate their malignant progression.

**HGF Promotes Neuroblastoma Expansion, Progression, and Tumor Angiogenesis *In vivo*.** To evaluate whether our *in vitro* data could be clinically relevant, we examined the ability of HGF to stimulate invasiveness and progression of human neuroblastoma cells *in vivo*. We inoculated neuroblastoma cells onto the chorioallantoic membrane of chick embryos. Because the chorion represents an epithelial barrier, only tumor cells with high invasive potential are

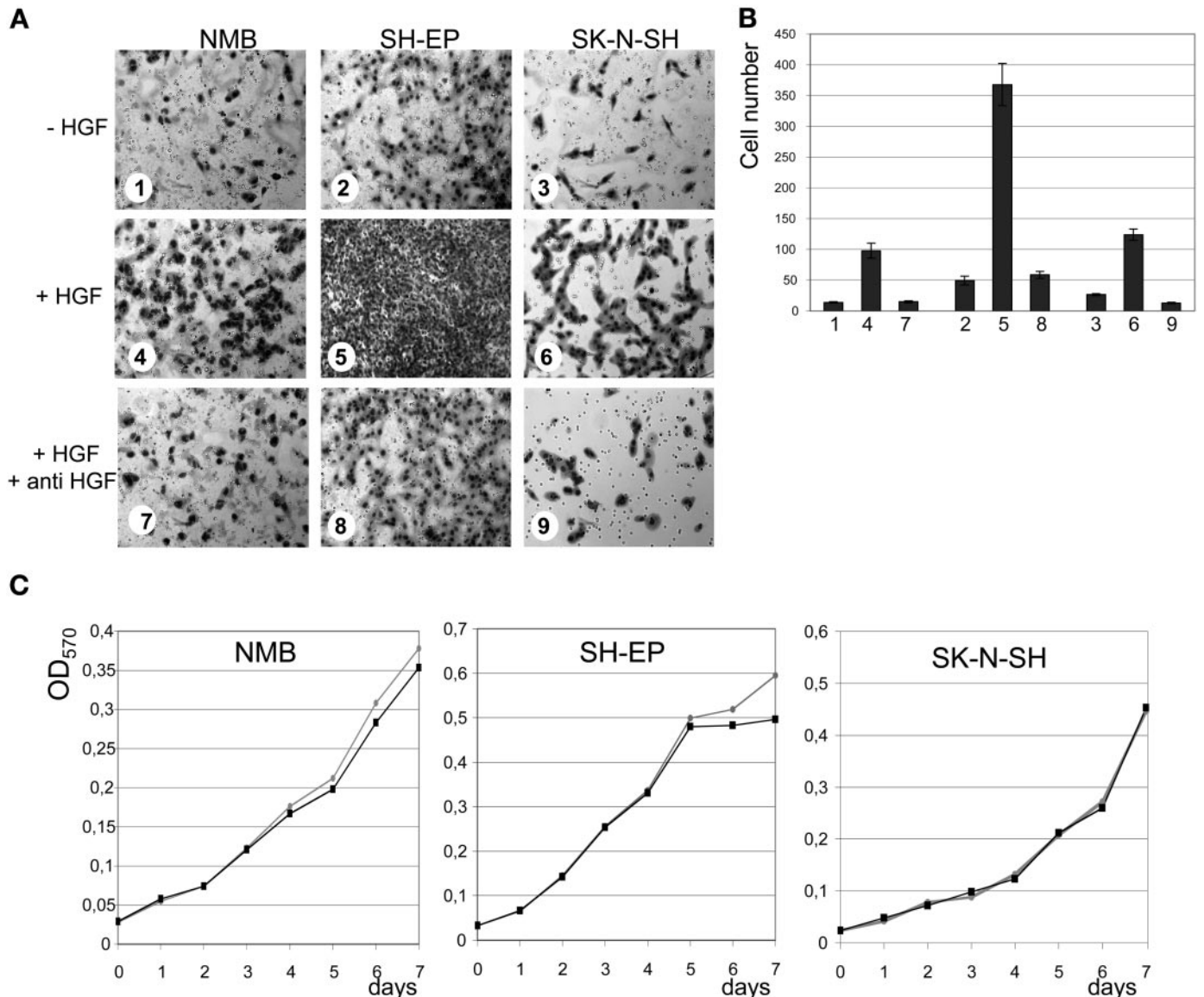


Fig. 3. Effect of HGF on invasion of neuroblastoma cell lines in Matrigel invasion chambers. **A**, Neuroblastoma cell lines were seeded in invasion chambers coated with Matrigel as described in Materials and Methods. Representative photographs taken at magnification  $\times 200$  show cells invading Matrigel and passed across the pored membrane. 1 through 3, nonstimulated cells; 4 through 6, cells treated with recombinant human HGF at 100 ng/mL; 7 through 9, idem D-F + anti-HGF (40  $\mu\text{g}/\text{mL}$ ). **B**, quantification of invading cells. Cells that have transversed the filter were stained and counted by light microscopy with magnification  $\times 400$  in 10 representative fields per well. Each value represents the mean of three independent experiments. **C**, growth rate of NMB, SH-EP, and SK-N-SH cells in the absence (●) or presence (■) of HGF (100 ng/mL). A colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay was performed after the indicated times. Values are the mean of triplicates. SDs were less than 10%.

able to cross this barrier and to get access to the chorionic vessels. In the absence of exogenous HGF, SH-EP neuroblastoma cells rarely formed solid tumors on the chorioallantoic membrane (Fig. 7A, 1). In only 2 of 14 eggs, we found very small tumors. However, in the presence of HGF, SH-EP cells readily penetrated the chorioallantoic membrane and generated multiple large tumors in 12 of 14 eggs (Fig. 7A, 2–3). The specific inhibitors of the MAPK cascade, PD98059 and U0126, and the specific phospholipase C- $\gamma$  inhibitor, U73122, were able to suppress HGF-mediated neuroblastoma cell invasiveness and tumor formation (Fig. 7A, 4–6), demonstrating that both of the c-Met-dependent signaling pathways are involved in these processes.

HGF is known to stimulate angiogenesis (40, 41). In accordance with this notion, the experimental neuroblastomas grew beyond critical angiogenesis-dependent sizes (approximately 2–3 mm) and reached diameters of up to 15 mm. In addition, the tumors were well vascularized and also harbored pathological, immature vessels, which gave rise to intratumoral hemorrhage (Fig. 7B), suggesting HGF-mediated angiogenesis in the

neuroblastomas. In fact, smooth muscle actin immunofluorescence within the tumor demonstrated numerous blood vessels (Fig. 7B, 3). Finally, we observed a strong staining of the tumors with antibodies directed against human neurofilament, indicating that the tumors represented graft-derived neuroblastomas (Fig. 7B, 2).

In conclusion, our results demonstrate the presence of a functional paracrine, rather than autocrine, HGF/c-Met signaling pathway in neuroblastoma cells, which mediates their invasiveness and progression. We also have shown that tumor progression can be blocked by specific inhibitors of this pathway. Therefore, targeting the HGF/c-Met signaling pathway may represent a therapeutic approach to improve the treatment of neuroblastoma-bearing patients.

## DISCUSSION

HGF is synthesized and released by mesenchymal cells during normal development. Its receptor c-Met is present on the surface of

Fig. 4. Expression and HGF-induced activation of MAPKs and phospholipase C- $\gamma$ , as well as constitutive activation of STAT3 in neuroblastoma cells. NMB, SH-EP, and SK-N-SH cells were serum starved for 18 hours and subsequently stimulated with HGF (60 ng/mL) for the indicated time points. Cell extracts were separated by SDS-PAGE (20  $\mu$ g/lane) and subjected to immunoblot analysis using specific antibodies against phospho-MAPKs, phospho-phospholipase C- $\gamma$ , and phospho-STAT3. All membranes were stripped and reprobbed with antibodies against the MAPKs p44/p42, phospholipase C- $\gamma$ , and STAT3 as a control for protein loading.

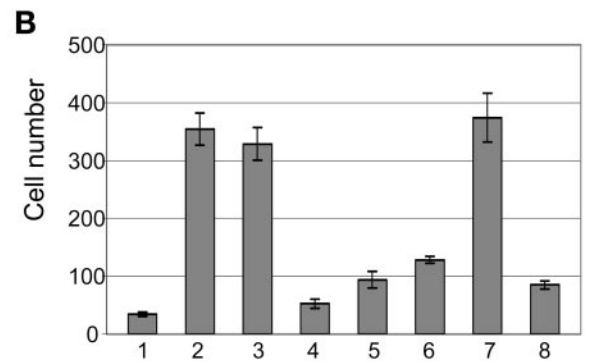
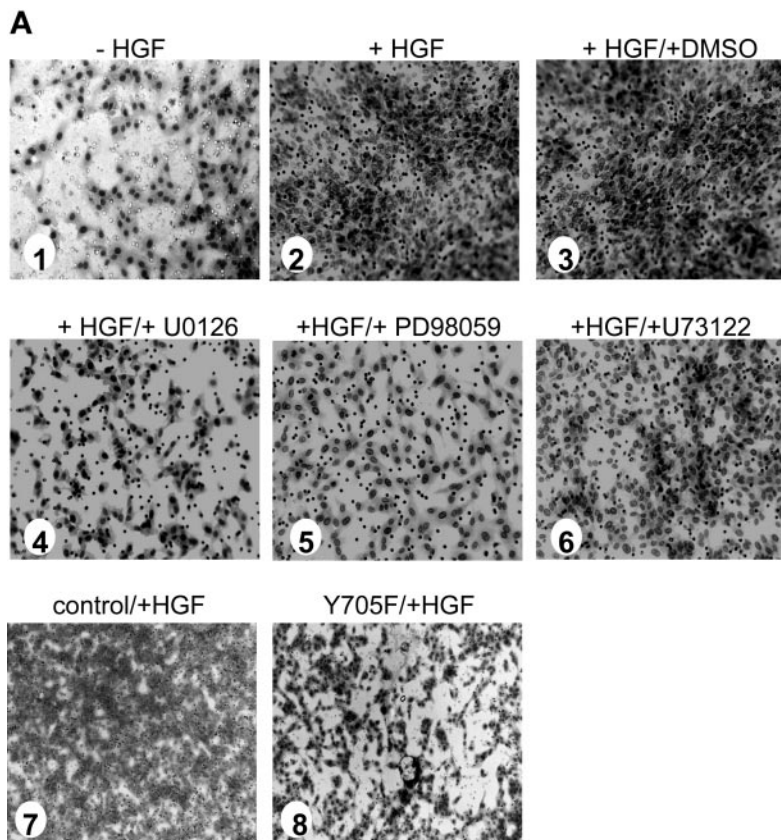
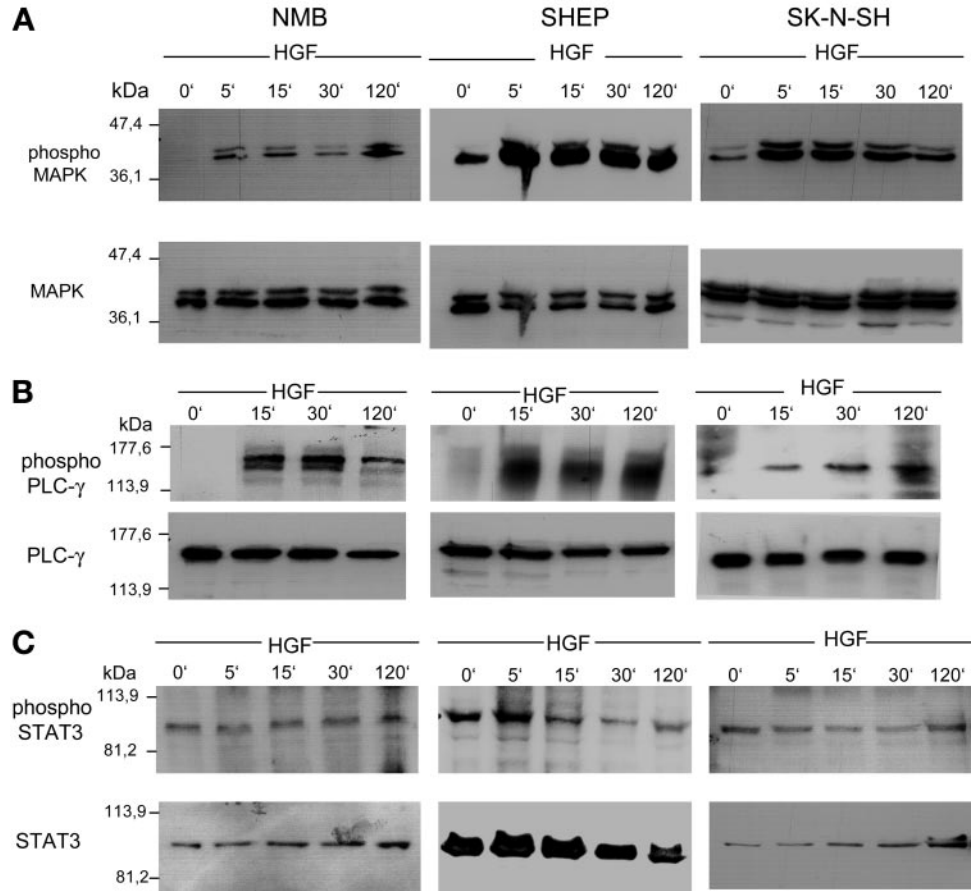


Fig. 5. Effect of specific inhibitors of MAPKs, of phospholipase C- $\gamma$ , and of dominant negative STAT3 on HGF-induced invasion of neuroblastoma cells in Matrigel. A. SH-EP cells were cultured for 36 hours in the absence (1) or in the presence of HGF (100 ng/mL; 2), HGF (100 ng/mL) plus solvent control (3), HGF (100 ng/mL) plus U0126 (20  $\mu$ mol/L; 4), HGF (100 ng/mL) plus PD98059 (30  $\mu$ mol/L; 5), or HGF (100 ng/mL) plus U73122 (2  $\mu$ mol/L; 6). In A, 7 and 8 show the invading SH-EP cells after transfection with the control vector or the dominant negative STAT3 mutant Y705F. Representative photographs were taken at magnification  $\times 200$  and demonstrate appearance of SH-EP cells invading the Matrigel and attaching to the lower side of the pored membrane (pore diameter = 8  $\mu$ m). B. The number of cells invading the Matrigel to the lower side of the membrane. Counting of invading cells occurred at magnification  $\times 400$  in 10 representative fields per well. Each value represents the mean of three independent experiments.

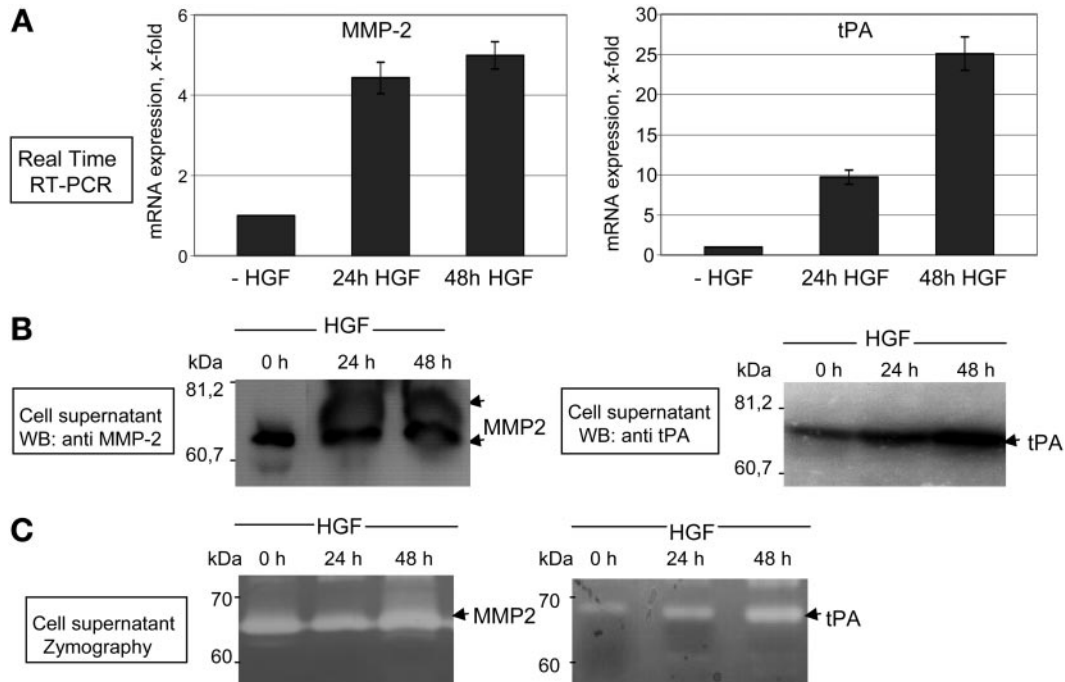


Fig. 6. Expression and activation of MMP-2 and tPA in HGF-stimulated neuroblastoma cells. A, real-time reverse transcription-PCR analysis of MMP-2 and tPA mRNA expression in SH-EP cells cultured for 24 and 48 hours in the absence and presence of HGF (60 ng/mL). B, expression of MMP-2 and tPA proteins in culture supernatants from SH-EP cells. Cells were grown to subconfluence and then serum deprived and stimulated or not with HGF (60 ng/mL). After 24 and 48 hours of HGF treatment, supernatants were processed for Western blot analysis. Aliquots derived from  $5 \times 10^4$  cells were resolved by SDS-PAGE on a 7.5% polyacrylamide gel and blotted with antibodies against MMP-2 and tPA. Arrows indicate the active ( $M_r$  66,000) and latent ( $M_r$  72,000) form of MMP-2 and the tPA protein ( $M_r$  68,000). C, zymographic analysis of culture supernatants from SH-EP cells. Cells were grown and stimulated with HGF as described above for Western blot analysis. Supernatant aliquots derived from  $5 \times 10^4$  cells were used for zymography in 10% polyacrylamide gels containing gelatin or casein. Zymographic analysis for assaying MMP, uPA, and tPA activity was performed as described in Materials and Methods.

epithelial and endothelial cells. By interacting with c-Met, HGF regulates cellular functions such as scattering, migration, and proliferation thereby contributing to essential morphogenetic processes (reviewed in ref. 20). Apart from normal development, c-Met has been found on the surface of malignant cells of epithelial origin, and HGF can trigger their invasive potential (reviewed in 32 and 42). Although this is well confirmed in tumors of adults, it was as yet unknown whether the HGF/c-Met signaling pathway plays a role in the progression of neuroblastomas. In the present study, we have demonstrated for the first time that HGF confers to neuroblastoma cells an invasive behavior *in vitro* and *in vivo*, which is central to their malignant progression.

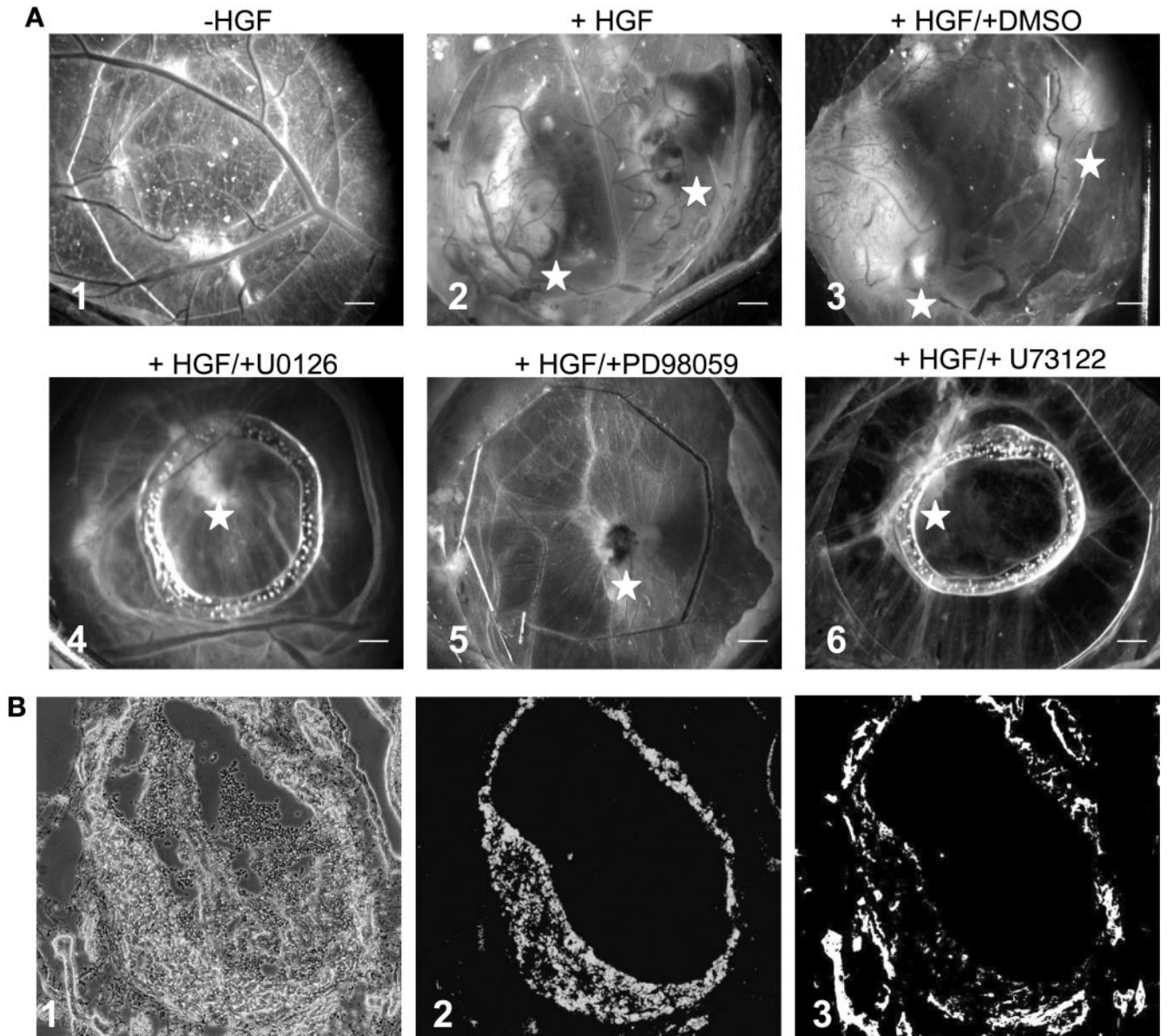
We conducted our studies because initial data revealed that c-Met is expressed in four of nine neuroblastoma-derived cell lines, suggesting expression and biological relevance of c-Met in human neuroblastomas *in vivo*. We further assumed that HGF derived from tumor stroma or adjacent normal tissue might interact with the c-Met receptor on neuroblastoma cells in a paracrine manner thereby stimulating tumor invasion. This is supported by reports suggesting that the stroma from various tumors of adults contains substantial amounts of HGF (43, 44).

We have examined HGF expression in the four c-Met-expressing cell lines NMB, SH-EP, SK-N-SH, and Kelly. All of them express HGF, but there is little or no basal c-Met receptor phosphorylation. Moreover, relevant tyrosine phosphorylation of c-Met occurred only after stimulation with exogenous HGF. The amount of HGF produced by neuroblastoma cells seems not to be sufficient to induce c-Met signaling. This indicates the existence of paracrine, rather than autocrine, HGF/c-Met loops in human neuroblastomas. Our assumption is supported by reports demonstrating that high concentrations of endogenous HGF are required to induce c-Met signaling (45). On the other hand, tumors of epithelial origin are known to use both paracrine

and autocrine HGF/c-Met signaling mechanisms (23, 24, 46, 47). It therefore can not be ruled out that some neuroblastomas may be able to produce HGF in considerable amounts and generate a functional autocrine HGF/c-Met loop. Thus, both paracrine and autocrine HGF/c-Met signaling pathways may exist in neuroblastomas *in vivo*.

Among many biological activities, invasiveness appears to be the most prominent cellular event induced by HGF, obviously requiring the activation of multiple c-Met-dependent pathways and a fine-tuned balance between intensity, duration, and synchrony of activated signaling molecules (reviewed in ref. 48). Deciphering such signaling profiles in neuroblastoma cells could provide a means to explore new drugs for the therapeutic suppression of the invasion of neuroblastoma into adjacent tissues.

In epithelial cells, the Ras/MAPK cascade is one of the major pathways activated by HGF/c-Met (14, 27). In this context, the duration of the activation of the MAPKs ERK1/ERK2 is a critical regulator of cellular behavior. Potempa and Ridley (49) have shown that the sustained MAPK activation induced by HGF is necessary for the disassembly of adherens junctions in epithelial cells, a critical step of cell dissociation before migration. Here, we have demonstrated that HGF induces a sustained (>2 h) activation of the MAPKs ERK1/ERK2 in NMB, SH-EP, and SK-N-SH neuroblastoma cells. This suggests that the HGF-mediated prolonged activation of ERK1/ERK2 stimulates the migratory rather than the proliferative potential of neuroblastoma cells. This notion is supported by our results, which show that HGF does not exert a mitogenic effect on neuroblastoma cells, but stimulates their invasiveness. The specific MAPK inhibitors U0126 and PD98059 suppress the HGF-induced invasiveness *in vitro* and *in vivo*, whereas proliferation or cell viability is not affected (data not shown). These findings raise the possibility that PD98059, U0126, or related compounds may be of clinical value in reducing the inva-



**Fig. 7.** Effect of HGF on invasion and tumorigenesis of neuroblastoma cells on the chick chorioallantoic membrane (CAM). **A.** Grafting of neuroblastoma tumors on the chorioallantoic membrane was performed as described in Materials and Methods. Three days before harvesting and every 2nd day during incubation on the chorioallantoic membrane, tumor cells were stimulated with HGF. Control cells remained unstimulated. Furthermore, we analyzed the effects of the inhibitors U0126, PD98059, and U73122 on tumor development on the chorioallantoic membrane. The bars represent 2 mm. **1,** no tumor growth in the absence of HGF. **2 and 3,** SH-EP neuroblastoma grown on the chick chorioallantoic membrane in the presence of HGF with (3) or without (2) solvent control. **4, 5, and 6,** SH-EP neuroblastoma grown on the chorioallantoic membrane in the presence of HGF plus U0126, PD98059, or U73122. **B.** phase-contrast overview and immunostaining of a representative cryosection. Representative photographs were taken at magnification  $\times 200$ . **1,** overview showing a tumor formed by HGF-stimulated SH-EP neuroblastoma cells on the chicken chorioallantoic membrane. Note the presence of a blood cyst in the middle of the tumor section. **2 and 3,** double staining of tumor cells and blood vessels with antibodies against neurofilament and smooth muscle actin.

siveness of neuroblastoma tumors in which this signaling cascade is constitutively activated.

Although the activation of the MAPK cascade appears to be central to HGF-mediated neuroblastoma cell invasiveness, other signaling molecules downstream of c-Met may as well be involved. Phospholipase C- $\gamma$  has been previously identified as a key molecule in HGF-mediated invasion of epithelial cells into collagen gels (28, 29). To study whether phospholipase C- $\gamma$  activation contributes to the HGF-induced invasiveness of neuroblastoma cells, we incubated the cells with HGF in the presence or in absence of the phospholipase C- $\gamma$  inhibitor U73122. Very similar to the MAPKs inhibitors tested here, U73122 inhibits HGF-triggered neuroblastoma cell invasiveness *in vitro*, and it suppresses the invasion and expansion of neuroblastoma cells into the chorioallantoic membrane *in vivo*. Thus, HGF-

stimulated neuroblastoma cell invasion is driven by phospholipase C- $\gamma$ -dependent pathways.

Studies on epithelial cells have shown that the integrity of the STAT3 pathway is essential for HGF-mediated invasion into collagen gels (16). Here, we show that STAT3 is constitutively active in c-Met-expressing neuroblastoma cell lines, suggesting abnormal STAT3 signaling. To elucidate its function, we transfected SH-EP cells with the STAT3 dominant negative mutant Y705F. Inhibition of the STAT3 signaling pathway substantially reduces neuroblastoma cell invasiveness *in vitro*, even in presence of HGF, whereas the behavior of control cells is not altered. Constitutive activity of STAT3 may be responsible for the basal invasiveness of NMB, SH-EP, and SK-N-SH cells, whereas activation of the MAPK cascade and phospholipase C- $\gamma$  are involved in HGF-triggered neuroblastoma cell invasiveness.



Proteolytic degradation of the extracellular matrix represents an early and critical step of tumor cell invasion into adjacent tissues. It is mediated by specific proteases including MMPs and the plasminogen activator system. We hypothesized that HGF promotes neuroblastoma cell invasiveness not only by activation of several signal transducers contributing to increased motility but also by triggering matrix degradation. We have examined the regulation and function of different MMPs and the plasmin system in neuroblastoma cells. Our results show increased expression and activity of MMP-2 and tPA, but not MMP-1, MMP-9, and uPA in HGF-stimulated cells. Our data are consistent with those of de Veas *et al.* (50), reporting that MMP-2 expression correlates with malignant neuroblastoma cell behavior *in vitro*, and those of Sugiura *et al.* (51), demonstrating expression of tPA in many human neuroblastomas *in vivo*. It appears possible that the tPA expression in primary neuroblastomas may in part be due to an active HGF/c-Met pathway. We were not able to detect uPA expression or activity in neuroblastoma cells studied. Other groups have reported that HGF induces activation of the uPA network, which correlates with enhanced tumorigenicity and invasiveness of human leiomyosarcoma and hepatocellular carcinoma cells (19, 52). Our data show that neuroblastoma cells may degrade the extracellular matrix by an alternate proteolytic system, *i.e.*, by activating tPA. In conclusion, we have shown that HGF activates proteolytic networks in neuroblastoma cells and contributes thereby to their invasive phenotype.

The chorioallantoic membrane invasion assay clearly supports this notion. The chorionic epithelium represents a barrier, which has to be degraded to get access to the chorioallantoic membrane vessels. The stroma of the chorioallantoic membrane may represent a supportive environment for neuroblastoma expansion. In fact, the HGF-stimulated neuroblastoma cells form large tumors *in vivo*, once they have penetrated the chorion. Because HGF has been reported to be a potent angiogenic factor both *in vitro* and *in vivo* (40, 41, 53), it may also contribute to neuroblastoma progression by stimulating angiogenesis. For example, HGF derived from various sources, the neuroblastoma cells, or the reactive stroma could promote angiogenesis in a paracrine manner by stimulating the migration and proliferation of adjacent vascular endothelial cells. The highly vascularized tumors grown on the chorioallantoic membrane in the presence of HGF suggest that HGF not only supports invasiveness but also contributes to an additional prognostically unfavorable feature of neuroblastomas, *i.e.*, angiogenesis.

In conclusion, our study indicates that the HGF/c-Met signaling pathway contributes in an essential manner to the progression of human neuroblastomas. Whether HGF and c-Met may represent independent prognostic factors in these tumors remains to be established. We have examined here whether HGF and c-Met expression correlates with known prognostic factors in neuroblastoma-derived cell lines. However, we found no correlation of HGF or c-Met with the prognostically unfavorable *N-Myc* or TrkB in the cell lines examined. Our data also show that targeting the HGF/c-Met pathway with inhibitors of the MAPK cascade and phospholipase C- $\gamma$  suppresses the HGF-mediated neuroblastoma cell invasion into the chorioallantoic membrane. Preliminary results obtained with the chorioallantoic membrane assay and neuroblastoma cells treated with neutralizing antibodies against HGF suggest additionally the therapeutic potential of targeting c-Met activation in neuroblastomas. Blocking the HGF/c-Met pathway, *i.e.*, by applying specific small molecule inhibitors of c-Met, could represent a means for an improved therapy of poor prognosis neuroblastomas.

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