Carcinogenesis vol.30 no.7 pp.1089–1096, 2009
doi:10.1093/carcin/bgp085
Advance Access publication April 9, 2009

c-Met activation in medulloblastoma induces tissue factor expression and activity: effects on cell migration

Mathieu Provençal[1], David Labbé[1], Ryan Veitch[1], Dominique Boivin[1], Georges-Étienne Rivard[2], Hervé Sartelet[3], Yves Robitaille[3], Denis Gingras[1] and Richard Béliveau[1,2,*]

[1]Laboratoire de médecine moléculaire, Université du Québec à Montréal, C.P. 8888, Succursale Center-ville, Montréal, Québec, Canada H3C 3P8, [2]Service d'Hématologie-Oncologie and [3]Département de Pathologie, Hôpital Sainte-Justine, 3175 Chemin Côte-Sainte-Catherine, Montréal, Québec, Canada H3T 1C5

*To whom correspondence should be addressed. Tel: +1 514 987 3000 ext. 8551; Fax: +1 514 987 0246;
Email: oncomol@nobel.si.uqam.ca

Met, the receptor for hepatocyte growth factor (HGF), is a receptor tyrosine kinase that has recently emerged as an important contributor to human neoplasia. In physiological and pathological conditions, Met triggers various cellular functions related to cell proliferation, cell migration and the inhibition of apoptosis, and also regulates a genetic program leading to coagulation. Since medulloblastomas (MBs) express high levels of tissue factor (TF), the main initiator of blood coagulation, we therefore examined the link between Met and TF expression in these pediatric tumors. We observed that stimulation of the MB cell line DAOY with HGF led to a marked increase of TF expression and procoagulant activity, in agreement with analysis of clinical MB tumor specimens, in which tumors expressing high levels of Met also showed high levels of TF. The HGF-dependent increase in TF expression and activity required Src family kinases and led to the translocation of TF to actin-rich structures at the cell periphery, suggesting a role of the protein in cell migration. Accordingly, addition of physiological concentrations of the TF activator factor VIIa (FVII) to HGF-stimulated DAOY cells promoted a marked increase in the migratory potential of these cells. Overall, these results suggest that HGF-induced activation of the Met receptor results in TF expression by MB cells and that this event probably contribute to tumor proliferation by enabling the formation of a provisional fibrin matrix. In addition, TF-mediated non-hemostatic functions, such as migration toward FVIIa, may also play a central role in MB aggressiveness.

Introduction

Medulloblastoma (MB) is the most common malignant tumor of the central nervous system (CNS) in children. These neuroepithelial tumors are thought to arise from neural stem cell precursors in the germinal cell layer of the cerebellum (1) and account for almost 20% of all intracranial tumors in children and for 40% of all childhood posterior fossa tumors (2).

Brain tumors are generally associated with worse prognoses than many other common pediatric cancers (3). An average of only 60% of affected children are cured and, furthermore, many survivors suffer from treatment-induced side effects (2). The classic treatment procedure for these tumors includes surgical resection, craniospinal radiations and chemotherapy; however, the main limitation of this strategy is its lack of specificity because it mainly relies on conventional cytotoxic therapies.

Abbreviations: CNS, central nervous system; Csk, C-terminal Src kinase; FVII, factor VII; HGF, hepatocyte growth factor; MB, medulloblastoma; PBS, phosphate-buffered saline; RTK, receptor tyrosine kinase; RT–PCR, reverse transcription–polymerase chain reaction; siRNA, small interfering ribonucleic acid; TF, tissue factor; TFPI, tissue factor pathway inhibitor.

Despite important advances in neuroimaging, diagnosis of MB is still mainly based on histological criteria; often, a major challenge for clinicians is to correlate the World Health Organization MB classification with biological behavior (4,5). Therefore, the necessity for improved risk stratification and clinical management requires a better understanding of the molecular mechanisms underlying MB growth and development (3,6).

A growing body of evidence indicates that altered regulation of receptor tyrosine kinases (RTKs) is frequent in epithelial human cancer and CNS tumors (7). Many RTKs have been identified as key components involved in MB progression and their expression levels are used as indicators of prognosis (8). For instance, neurotrophin-3 receptor (tropomyosin-related kinases C) was the first RTK found to be associated with clinical significance in CNS tumors and its expression, as well as that of fibroblast growth factor receptor, is a favorable prognostic indicator in MB (9,10). In contrast, ErbB2 (HER2/Neu) (11), PDGFRb (12,13) and insulin-like growth factor receptor (14) are linked to an unfavorable prognosis.

Recently, the scatter factor/hepatocyte growth factor (HGF):Met pathway has emerged as an important contributor to human neoplasia (15). Met has also been added to the list of RTKs associated with poor prognosis in human MB (16). Met affects tumorigenicity and malignant progression by inducing cell cycle progression (17), tumor cell migration, invasion and metastasis (18,19) and tumor angiogenesis (20). Furthermore, Met activation leads to the expression of proteins such as matrix metalloproteinases (21,22) and vascular endothelial growth factor (23), which are known for their important roles in tumor promotion. It has also been shown that the *Met* oncogene also drives a genetic program linking cancer to hemostasis, principally by promoting PAI-1 and Cox-2 protein expression (24).

Blood coagulation disorders, also referred as coagulopathies, have been known to be associated with cancer for many years; however, the molecular basis of this relationship is not clearly understood (25). Nearly 50% of all patients with malignant disease, and up to 90% of those with metastatic lesions, suffer from these coagulopathies (26). Among these disorders, thrombosis is one of the major complications, occurring in 10–15% of cancer patients (27). Better known as Trousseau's syndrome, these thrombotic events are often the first clinical manifestations of an occult malignancy (28). This procoagulant state of tumors is due to the expression of specific proteins such as tissue factor (TF).

TF, a class 2 cytokine receptor, is a 47 kDa transmembrane glycoprotein that consists of three domains: a large extracellular domain; a transmembrane segment and a short cytoplasmic tail (29). TF was first identified as the main trigger of the extrinsic pathway of coagulation. The process of clot formation is considered to be a two-stage process and the initiation phase of coagulation begins when disruption of vessel walls exposes TF to circulating factor VII (FVII). Ensuing interaction between TF and FVII induces a conformational change in the protease domain of FVII, allowing its activation into FVIIa. The newly form TF:FVIIa activates FIX and FX, leading to the propagation phase of coagulation and the formation of a fibrin deposit (29). Aside from its procoagulant action, TF can act through intracellular and extracellular signaling to stimulate cell migration (30), therefore contributing to the cancer metastasis process (31,32). TF also regulates angiogenesis through signaling by its cytoplasmic domain (33). However, despite all this knowledge about TF and its role in general cancer development, little is known about the mechanisms of TF's expression and contribution in MB.

In this work, we report that stimulation of DAOY with HGF leads to a marked increase of TF expression in these cells. This TF expression is mediated by a Src-dependent mechanism and this increased expression contributes to an upregulated TF activity, thus inducing a procoagulant state. These *in vitro* experiments are supported by further results describing correlation between Met and TF expression in clinical tumor

specimens. Furthermore, TF is also involved in non-hemostatic functions such as cell migration toward FVIIa, its natural ligand.

Materials and methods

Materials

Cell culture media were obtained from Wisant (St-Bruno, Quebec, Canada) and sera were purchased from Hyclone Laboratories (Logan, UT). Recombinant human growth factors (HGF, platelet-derived growth factor and insulin-like growth factor) were purchased from R&D Systems (Minneapolis, MN), whereas basic-fibroblast growth factor was obtained from Upstate Cell Signaling (Lake Placid, NY) and epidermal growth factor was purchased from BD Biosciences Discovery Labware (Bedford, MA). The extracellular signal-regulated kinase-1/2 mitogen-activated protein kinase inhibitor (PD98059), p-38 mitogen-activated protein kinase inhibitor (SB203580), Src family kinase inhibitor (PP2), protein synthesis inhibitor (cycloheximide), transcription inhibitor (Actinomycin D) and the coagulation Factor X from human plasma were all purchased from Calbiochem (La Jolla, CA). c-Met-specific inhibitor (SU11274) was obtained from EMD Chemicals (Gibbstown, NJ). All products for electrophoresis and western blots were purchased from Bio-Rad (Hercules, CA). TF antibodies were obtained from American Diagnostica (Stamford, CT), whereas anti-Met and anti-phospho-Met monoclonal antibody were obtained from Cell Signaling Technology (Beverly, MA). Transfection reagents Polyfect and Lipofectamine™ 2000 were purchased from Qiagen and Invitrogen (Burlington, Ontario, Canada), respectively. TRIzol reagent and the superscript one-step reverse transcription–polymerase chain reaction (RT–PCR) kit were obtained from Invitrogen. FVIIa was kindly provided by Dr G.É.Rivard (Service d'Hématologie-Oncologie, Hôpital Sainte-Justine, Montréal, Quebec, Canada) and Factor Xa chromogenic substrate Biophen CS-11 (22) was obtained from Aniara Corporation (Neuville-sur-Oise, France).

Cell culture

MB cell lines (DAOY and D283) and glioblastoma cell lines (U-87 and U-138) were purchased from the American Type Culture Collection (Manassas, VA). All cells were cultured at 37°C under a humidified atmosphere containing 5% CO2. DAOY, U-87 and U-138 cells were maintained in Eagle's modified minimum essential medium and supplemented with 10% (vol/vol) bovine calf serum, 4 mM glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin. D283 cells were cultured in Eagle's modified minimum essential medium containing 10% (vol/vol) fetal bovine serum, 4 mM glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin.

Cell stimulation with HGF and membrane isolation

DAOY, D283, U-87 and U-138 cells were grown to near confluence in 100 mm petri dishes and starved for 48 h in serum-free media. In time-course experiments, cells were stimulated from 1 to 12 h at 37°C with 50 ng/ml HGF. Otherwise, cells were stimulated 6 h with 50 ng/ml HGF, epidermal growth factor, platelet-derived growth factor and insulin-like growth factor or 30 ng/ml basic-fibroblast growth factor. Cells were then washed with 4 ml of lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM sodium orthovanadate and 1 mM sodium fluoride) (buffer A) and scraped with 2 ml of buffer A. For the isolation of cell membranes, cells were resuspended in 500 µl of buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM sodium orthovanadate and 1 mM sodium fluoride) (buffer B) and homogenized twice using a Polytron for 30 s at 50% amplitude. Cells were then centrifuged at 4000 r.p.m. for 5 min at 4°C and the resulting supernatants were centrifuged again at 55 000 r.p.m. for 60 min at 4°C. Pellets were resuspended in 50 µl of buffer B. Protein concentrations were determined by the bicinchoninic acid method (Pierce, Canada, ON).

RNA isolation and RT–PCR

Total RNA was isolated from DAOY using TRIzol reagent according to the manufacturer's instructions. One microgram of total RNA isolated from cells was amplified with Superscript™ One-step RT–PCR with Platinum® TAQ, using specific primers annealing to human TF (sense, 5′-CCCGTCAATCAAGTCTACACTGTTC-3′ and antisense, 5′-TTGGCTGTCCGAGGTTTGTCTC-3′) and human tissue factor pathway inhibitor (TFPI) (sense, 5′-CATCAGAGATTTTACTTAGATGA-3′ and antisense, 5′-CATTGCTATAACAAATTCACA-3′).

Western blotting procedures

Identical amounts of protein from each sample were prepared in Laemmli sample buffer (125 mM Tris–HCl, pH 6.8, 20% glycerol, 4% sodium dodecyl sulfate, 10% b-mercaptoethanol and 0.00125% bromophenol blue), boiled for 5 min and resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Proteins were transferred onto polyvinylidene difluoride membranes and blocked overnight at 4°C with tris buffered saline-tween buffer (20 mM Tris–HCl, pH 7.5, 147 mM NaCl, 0.1% Tween 20) containing 3% bovine serum albumin or 5% milk. Membranes were incubated with the specific primary antibody for 1 h at room temperature. Immunoreactive bands were revealed following 1 h incubation with horseradish peroxidase-conjugated anti-mouse, anti-rabbit or anti-goat antibodies, and the signals were visualized using an enhanced chemiluminescence detection system.

TF activity

Cell membrane samples were assayed for TF activity using a chromogenic assay. Each sample was incubated with FVIIa (10 nM) and Factor Xa chromogenic substrate Biophen CS-11 (800 µM) in cell buffer (21 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.4, 137 mM NaCl, 5 mM KCl, 0.75 mM Na_2HPO_4, 11 mM glucose, 2 mM $CaCl_2$). The reaction was started by the addition of FX (1 µM), and FXa generation was measured by spectrophotometry at 37°C for 1 h at 405 nm, as described earlier (34).

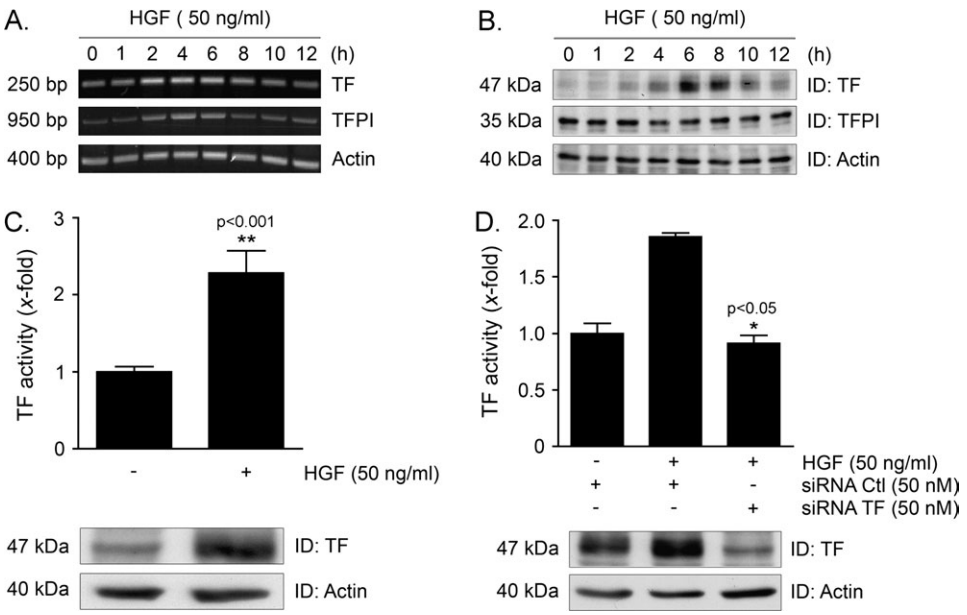

Fig. 1. HGF induces TF expression and activity in DAOY. Confluent DAOY in 100 mm dishes were serum starved for 48 h and then stimulated with 50 ng/ml HGF for 1–12 h. (**A**) Total RNA was isolated as described in Materials and Methods and amplified by RT–PCR. Actin was used as control. (**B**) Equal amounts of protein from cell lysates were subjected to electrophoresis and TF and TFPI protein levels were visualized by western blot. (**C**) DAOY was stimulated with HGF for 6 h and procoagulant activity was measured using a chromogenic assay, as described in Materials and Methods. (**D**) DAOY was transfected with either a control or with a TF-specific siRNA prior to stimulation with HGF and the extent of procoagulant activity was measured as described in (C).

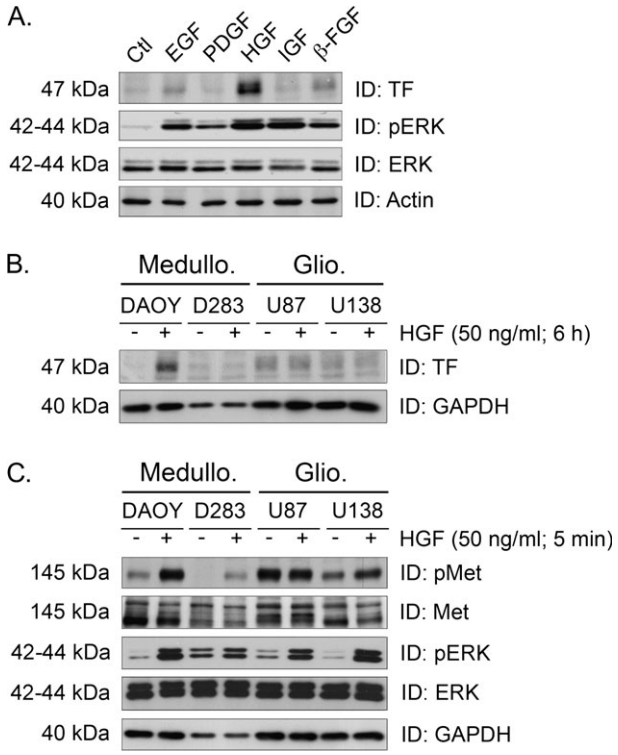

Fig. 2. Met is responsible for TF expression in DAOY MB cells. (**A**) Confluent DAOY in 100 mm dishes were serum starved for 48 h, stimulated with growth factors [epidermal growth factor (EGF), platelet-derived growth factor (PDGF), HGF, insulin-like growth factor (IGF) and basic-fibroblast growth factor] and TF expression was visualized by western blot. (**B**) Two MB cell lines (DAOY and D283) and two glioblastoma cell lines (U-87 and U-138) were grown to confluency and serum starved for 48 h. Cells were stimulated with 50 ng/ml HGF for 6 h and TF expression was visualized by western blot (**C**) Confluent DAOY, D283, U-87 and U-138 cells were serum starved for 48 h. Cells were then stimulated with 50 ng/ml HGF for 5 min. Equal amounts of protein from cell lysates were subjected to electrophoresis and western blotting. Levels of TF, phospho-Met, Met, phospho-extracellular signal-regulated kinase (pERK) and extracellular signal-regulated kinase (ERK) were detected using specific antibodies.

Plasmid transfection method

The plasmid encoding wild-type Src was kindly provided by Dr Isabelle Royal (Université de Montréal, Montréal, Canada). The plasmid encoding C-terminal Src kinase (Csk) was kindly provided by Dr. H.Hanafusa (Osaka Bioscience Institute, Japan) and the Csk coding sequence was subcloned at the EcoRI site of pcDNA3.1. Transient transfection of the plasmids (4 μg) in subconfluent DAOY was performed using the polyfect reagent according to the manufacturer's instructions (Qiagen). Cells were starved overnight 24 h posttransfection and then stimulated with 50 ng/ml HGF for 6 h.

Small interfering ribonucleic acid transfection method

Small interfering ribonucleic acid (siRNA) (hTF167i) targeting human TF messenger RNA and control siRNA were purchased from Qiagen. siRNA TF target sequences are sense, r(GCGCUUCAGGCACUACAAA)dTdT and antisense, r(UUUGUAGUGCCUGAAGCGC)dTdT, as described previously (35). Cell medium of subconfluent DAOY was replaced by serum- and antibiotics-free Eagle's modified minimum essential media. Meanwhile, TF or control siRNA (50 nM each) was mixed with 30 μl of lipofectamine™ 2000 reagent and incubated for 20 min at room temperature. After this incubation, the mix was added to the cells for 6 h at 37°C. After this period, cell medium was replaced with media containing 10% bovine calf serum as described in the previous cell culture section.

Cell migration assays

Prior to the migration assay, cells (DAOY) were stimulated 6 h with 50 ng/ml HGF as described above. Transwell inserts (8 μm pore size; Costar, Cambridge, MA) were precoated with 0.15% gelatin–phosphate-buffered saline (PBS) by adding 600/100 μl in the lower/upper chambers for 24 h at 4°C. The Transwells were then washed with PBS and assembled in 24-well plates. The upper chamber of each Transwell was filled with 100 μl of cells (50 000 cells/chamber) and the cells were allowed to adhere for 45 min. Migration was initiated by adding FVIIa (15 nM) to the lower chamber. The plate was placed at 37°C in 5% CO_2–95% air for 3 h. Cells that had migrated to the lower surface of the filters were fixed with 10% formalin phosphate and stained with 0.1% Crystal Violet–20% methanol (vol/vol). The migration was quantified using computer-assisted imaging and data are expressed as the average density of migrated cells per four fields (×50 magnification).

Immunofluorescence and confocal microscopy

Confocal microscopy studies were done as described previously (36). Briefly, DAOY was seeded onto coverslips coated with 0.15% gelatin–PBS in a 24-well plate and were serum starved for 24 h in media containing 0% bovine calf serum. The cells were then stimulated for 6 h with 50 ng/ml HGF. After this stimulation, DAOY was fixed in 3.7% formaldehyde for 15 min, permeabilized with 0.2% Triton X-100 for 5 min and stained with a 1/2500 dilution of Texas Red phalloidin (Sigma, Canada, ON) for 30 min. Non-specific sites were blocked with 1% bovine serum albumin in buffered saline containing 0.1% Tween 20 for 30 min and cells were stained with primary antibodies against TF (1/150) for 30 min at room temperature. Cells were then incubated with Alexa488-conjugated secondary antibody (for TF staining) and slides were mounted with ImmunoFluor Mounting Medium (MP Biomedicals, Canada, Que). Immunostaining was visualized and photographed using a Zeiss LSM 510 Meta confocal microscope.

Immunohistochemistry

The 18 human MB specimens used in the current study were obtained surgically from patients at Hôpital Sainte-Justine between 1996 and 2007, as described previously (37). These patients were all diagnosed with classic MB. The surgical specimens were fixed in 10% formalin and embedded in paraffin. Thin tissue sections were dewaxed on a poly-L-lysine coated slide with xylene and rehydrated with a graded series of ethanol at room temperature. Blocking of endogenous peroxidase activity was carried out by immersing the tissue section in 0.3% methanolic hydrogen peroxide for 15 min at 4°C. Primary monoclonal antibodies for TF and Met were applied at a dilution of 1:100 and incubated overnight at 4°C. After three washes with PBS, the peroxidase-labeled mouse secondary antibody was applied. After three washes with PBS, the tumor sections were incubated with 3-amino-9-ethylcarbazole in 0.1 M sodium acetate (pH 5.2) for 30 min at room temperature. After three PBS washes, the sections were counterstained with hematoxylin for 10 min and then rinsed extensively with distilled water. Sections incubated with normal serum instead of primary antibodies served as controls. The intensity of TF and Met staining was classified into five levels as follows: (−), negative; (+), 0–25% of positive cells; (++), 25–50% of positive cells; (+++), 50–75% of positive cells; (++++), 75–100% of positive cells.

Statistical analysis

The data are presented as means ± SEMs and statistical analyses were performed with Student's *t*-test where one group was compared with the control group.

Results

HGF stimulates TF expression and activity in MB

Previous reports have suggested that the *Met* oncogene may control a genetic program linking cancer to hemostasis (24). In order to determine if Met activation by HGF could stimulate TF expression, a time-course experiment was performed on DAOY cells. As shown in Figure 1A, RT–PCR studies demonstrated that the stimulation of MB cells by 50 ng/ml HGF from 0 to 12 h caused an increase in messenger RNA levels for both TF and TFPI, the natural inhibitor of TF. However, at the protein level only TF showed a significant increase, whereas TFPI protein expression remained steady throughout the time course (Figure 1B). To further explore whether TF expression correlated with increased procoagulant activity, the cells were stimulated with HGF for 6 h and the extent of TF activity was measured by the FVIIa-dependent generation of Factor Xa activity. As shown in Figure 1C, HGF induced a marked increase in the cells' procoagulant activity, this increase being mediated by TF as reflected by the marked inhibitory effect of a siRNA specific for this protein (Figure 1D). Overall, these data suggest that, following stimulation with HGF, DAOY expresses TF and thus exhibits a procoagulant state.

Over the years, many reports have pointed out that RTK deregulation plays a pivotal role in cancer (7). For this reason, we next stimulated DAOY with different growth factors known to influence the

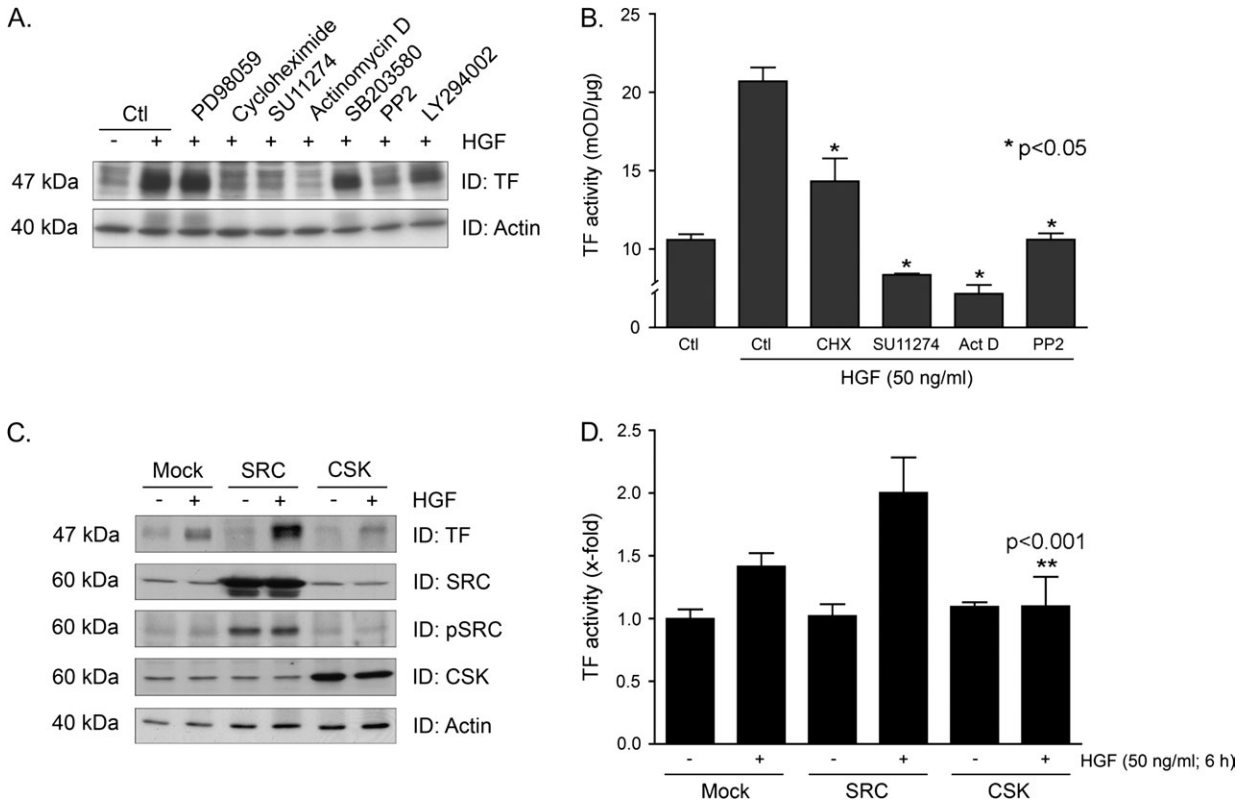

Fig. 3. TF expression and activity in DAOY is regulated by Met activation and Src family kinase. (**A**) Confluent and serum-starved DAOY were treated 1 h with different inhibitors at 37°C prior to HGF stimulation for 6 h. Equal amounts of protein from cell lysates were subjected to electrophoresis and TF protein expression was visualized by western blot. (**B**) TF activity was quantified as described in Materials and Methods. (**C**) DAOY was transiently transfected with Src and Csk. After a 24 h starvation, cells were stimulated with 50 ng/ml HGF for 6 h and TF expression was visualized by western blot. (**D**) TF activity was measured as described in Materials and Methods.

development of MB, and we monitored their effect on TF expression. As shown in Figure 2A, HGF was the only growth factor that induced an increase in TF expression, whereas all other growth factors tested were ineffective in spite of their stimulatory effects on other signaling cascades, such as extracellular signal-regulated kinase. Moreover, because other CNS tumors (i.e. glioblastoma) have been shown to express TF (38), we further tested the impact of HGF stimulation on two MB (DAOY, D283) and two glioblastoma (U-87, U-138) cell lines (Figure 2B). Surprisingly, western blot analysis revealed that only DAOY expresses TF when stimulated with HGF. This preferential effect was not related to the absence of the HGF/Met pathway in non-responsive cells since every cell line used in this experiment expressed a Met receptor that could be activated upon HGF stimulation (with the notable exception of the U87 cell line that showed ligand-independent activation of the receptor) (Figure 2C). These data thus suggest that TF expression induced by the HGF/Met-signaling pathway may represent a specific event occurring during the development of certain types of MB.

TF expression requires Met and Src family kinase activation

In order to preliminary determine the intracellular pathways involved in the transcription and expression of TF in MB cells, serum-starved DAOY was pretreated 1 h with mitogen-activated protein kinase inhibitors (PD98059, SB203580), transcription and protein synthesis inhibitors (actinomycin D, cycloheximide), a Met-specific inhibitor (SU11274) and a Src family kinase inhibitor (PP2) prior to HGF stimulation. As shown in Figure 3A and B, TF expression and activity was clearly inhibited by actinomycin D and cycloheximide, thus indicating that *de novo* protein synthesis is required following HGF stimulation. Furthermore, SU11274 and PP2 also inhibited TF expression and activity (Figure 3A and B), implying that TF protein expression requires proper activation of Met, which in turn signals through a Src family kinase-dependent pathway. To confirm the involvement of this pathway in TF expression, DAOY was transfected with Src or Csk, the endogenous inhibitor of Src, and cells were then stimulated with HGF, following that TF expression and activity were assessed as described above. As shown in Figure 3C, DAOY-overexpressing Src highly expressed TF when stimulated with HGF. In contrast, DAOY overexpressing Csk were less sensitive to HGF, and TF expression was repressed in these cells. Moreover, TF activity is also dependent on Src expression and activity, since cells transfected with Src showed a 2-fold increase in TF activity, whereas cells transfected with Csk showed no increased procoagulant activity (Figure 3D). Overall, these data strongly suggest that Src family kinases play an important role in the HGF-dependent increase in TF expression in DAOY.

HGF stimulates colocalization of TF and cortical actin in DAOY

We next sought to determine whether TF was randomly expressed in DAOY or whether it was colocalized with specific migration-related structures. As shown in Figure 4, serum-starved control cells showed a low level of TF expression and evenly distributed actin; merging the images did not reveal any colocalization (top three panels). In contrast, stimulation of cells with 50 ng/ml HGF for 6 h led to high cortical and peri-nuclear TF expression that colocalized with actin-rich structures at the cell periphery (indicated by white arrows). The Met-specific inhibitor (SU11274) and the Src family kinase inhibitor (PP2) both reduced TF and cortical actin expression to the levels found in unstimulated cells. These data imply that in DAOY, TF colocalizes with migration-related structures upon HGF stimulation and therefore suggest that TF may be involved in MB cell locomotion.

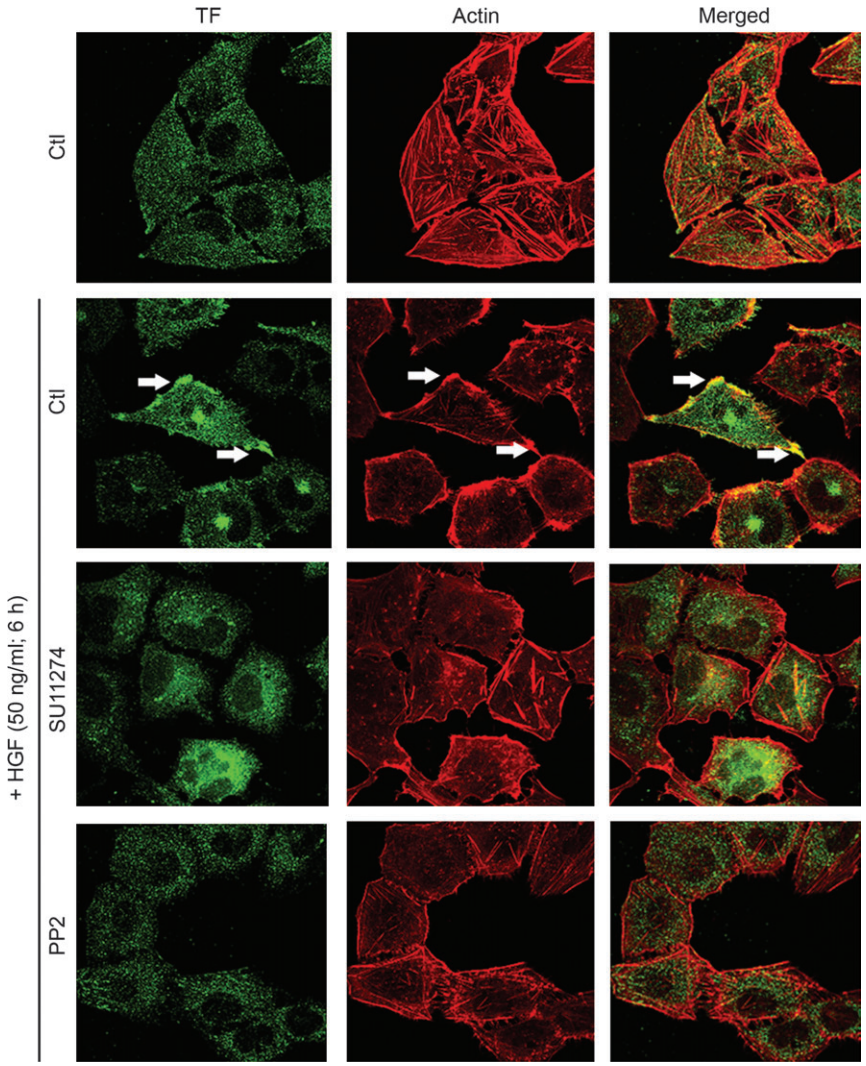

Fig. 4. HGF-stimulated DAOY exhibits a cortical expression of TF colocalizing with reorganized actin-rich structures. DAOY (3×10^4 cells/well) was seeded onto gelatin-coated coverslips in a 24-well plate. Cells were serum starved for 48 h and pretreated (or not) for 1 h with SU11274 (5 μM) or PP2 (10 μM) and stimulated (or not) with HGF for 6 h. Cells were fixed and double stained with Texas Red-conjugated phalloidin for actin and with a specific antibody against TF, followed by incubation with Alexa488 secondary antibody. Representative cell images were obtained by confocal microscopy, as described in Materials and Methods. Arrows indicate staining of TF and cortical actin (membrane ruffles) at the cell periphery of HGF-stimulated cells.

TF expression in DAOY enhances cell migration

Increasing evidence indicates that TF may be involved in cell migration through a non-hemostatic pathway (30). Therefore, we assessed whether HGF-dependent expression of TF favored cell migration toward its natural ligand, FVIIa. As shown in Figure 5A, cells that had been stimulated with 50 ng/ml HGF for 6 h clearly expressed increased levels of TF and had the ability to migrate toward a physiological concentration of FVIIa (15 nM). Cells transfected with a TF siRNA were unresponsive to HGF stimulation and unable to migrate toward FVIIa compared with cells transfected with the control siRNA, further highlighting the essential role of TF in this process (Figure 5B). In order to validate the role of Met and Src activation in this process, the Met inhibitor (SU11274) and the Src family kinase inhibitor (PP2) were used (Figure 5C). As expected, both inhibitors significantly reduced both TF expression levels and the number of migrating cells.

TF and Met expression levels correlate in clinical MB tumor specimens

Immunohistochemical staining with the monoclonal antibody against human TF (CD 142) and the monoclonal antibody against Met was carried out to evaluate the levels of TF and Met expression in clinical tumors. TF antibody was strongly immunoreactive in 8 of the 18 tumor specimens. The TF antibody labeled cancer cells as well as smooth muscle cells underlying tumor blood vessels. We found that six of the eight TF-positive tumors also showed a strong Met immunoreactivity (Figure 6), strongly suggesting that high TF expression correlated with high levels of Met in human tumors. Further exploration of the clinical database revealed that the six tumors exhibiting high TF and Met expression also exhibited the highest vascular proliferation and proliferative index, ranging from 40 to 90%. Furthermore, among the six tumors expressing high levels of TF and Met, one tumor showed evidence of leptomeningeal invasion. Together these results suggest that tumors expressing high levels of both TF and Met are probably those with the greatest chances of evolving rapidly and forming distant metastases.

Discussion

Met was recently identified as a marker of poor prognosis in MB (16), as well as an important molecular link between cancer and hemostasis (24). In this study, we show that in DAOY, a well-established *in vitro* model of metastatic MB, the HGF/Met pathway plays a major role in increased TF expression, conferring these malignant cells with

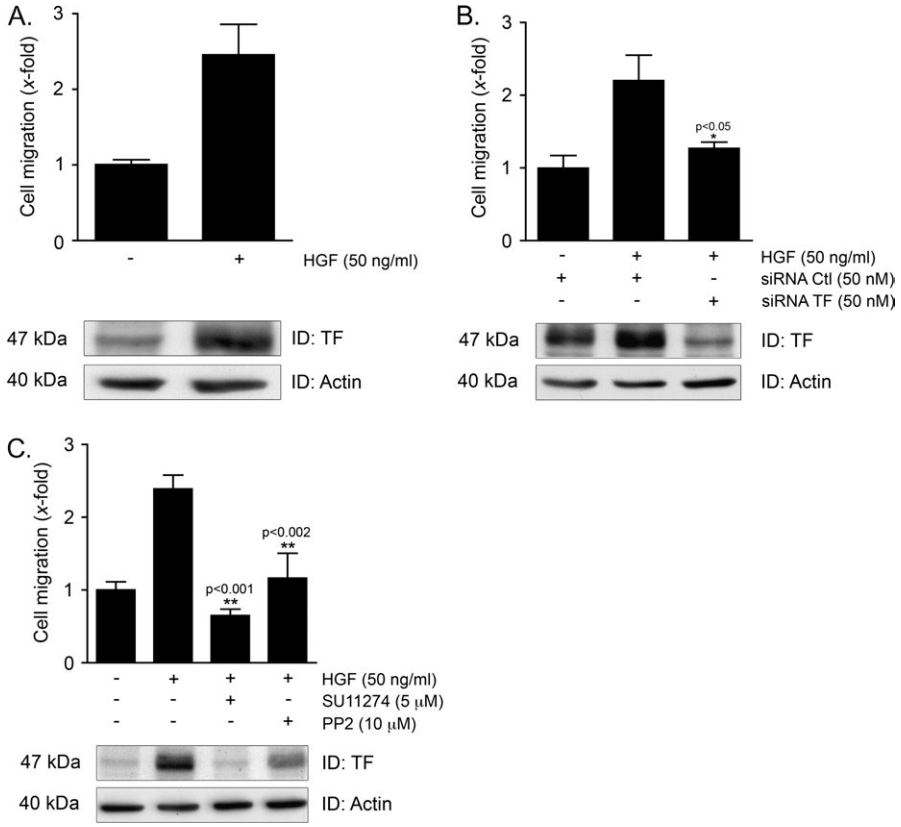

Fig. 5. TF expression in DAOY enhances cell migration toward Factor VIIa. (**A**) Control or HGF-stimulated DAOY was allowed to attach to filters and migrate for 3 h in serum-free media containing 15 nM FVIIa. (**B**) In order to silence TF expression, DAOY was transfected with a control or a siRNA specific to TF prior to stimulation with HGF and cells were subjected to a migration assay for 3 h in serum-free media containing 15 nM FVIIa. (**C**) One hour prior to HGF stimulation, DAOY was pretreated with SU11274 (5 μM) and PP2 (10 μM). Cells were then allowed to attach to filters and migrate in the presence of 15 nM FVIIa. In all cases, cell migration was quantified using a computer-based program as described in Materials and Methods. Data are expressed as *x*-fold induction ± SD of non-stimulated controls.

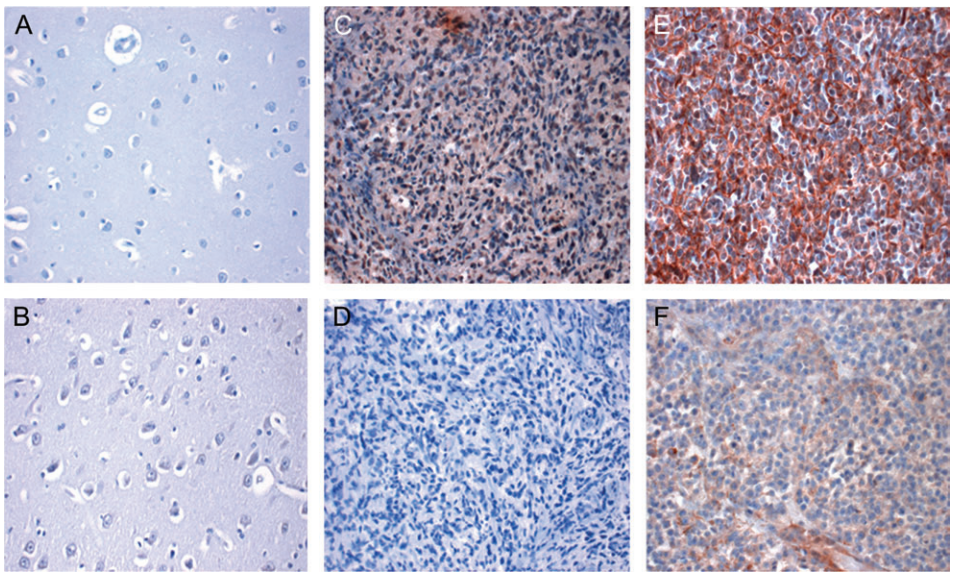

Fig. 6. Immunohistochemistry of TF and Met proteins in human MB surgical specimens. (**A** and **B**) Controls prepared as described in Materials and Methods. (**C** and **D**) Tumors expressing low levels of TF (+) and Met (−), respectively. (**E** and **F**) Tumors expressing high levels of both TF (++++) and Met (+++), respectively. The examples shown were obtained from the same tumors. (×400).

a procoagulant and migratory phenotype. To the best of our knowledge this is the first report establishing a direct link between Met activation and TF expression in cancerous brain cells. Interestingly, this relationship is not restricted to cell lines grown *in vitro* since we observed a strong correlation between Met and TF expression levels in human MB tumor specimens.

HGF was the only growth factor found to influence TF synthesis in *in vitro* experiments. Upon stimulation, TF production in MB requires *de novo* protein synthesis, instead of being part of a recycling process from an intracellular pool. Further investigation revealed that the Src family kinases, shown previously to be involved in downstream signaling events mediated by Met (39), are needed for expression and activity of TF. While TF is upregulated upon HGF stimulation, the expression level of its natural inhibitor, TFPI, remains steady. Such an imbalance between hemostatic activator and inhibitor thus probably contribute to the establishment of the procoagulant state in HGF-stimulated cells (25).

Surprisingly, DAOY seems to exhibit a unique HGF/Met-dependent mechanism of TF expression when compared with other MB or glioblastoma cells, even though all these cell types express similar levels of TF under basal conditions (data not shown) and possess functional Met receptors. The mechanisms underlying this differential sensitivity to HGF remain unclear but may be related to the heterogeneity of cell populations observed in CNS tumors, reflecting the diverse origins of MB malignant cells (5). Furthermore, other cellular pathways may contribute to TF expression in different tumors. For example, in glioma, other investigators reported that TF expression is regulated by other cellular circuitry, including downregulation of PTEN, activation of early growth response gene-1 (Egr-1) and paracrine stimulation by vascular endothelial growth factor secreted by tumor endothelial cells (40–42).

In our study, TF-expressing cells demonstrated a significant increase in their migratory potential toward a physiological concentration of FVIIa, thus implying a role for TF in the invasion and metastasis processes, hallmarks of cancer aggressiveness. Moreover, to fulfill its chemotactic function, we observed that TF is not just randomly expressed in the cell but rather associates at the cell surface with actin-rich membrane ruffles. Others investigators have reported that the formation of these cortical actin structures was mediated by a Src family kinase (43) and also that Src activation by Met mediates metastatic properties of colorectal carcinoma cells (44). These finding corroborate our results because both PP2, a Src family kinase inhibitor, and SU11274, a Met-specific inhibitor, inhibited TF expression and the formation of actin-rich ruffles at the cell periphery.

Overall, the acquisition of a procoagulant phenotype by MB may provide to the tumor a fibrin-rich environment, which is commonly observed in primary and metastatic human brain tumors (45). Fibrin deposits protect cancer cells from the immune system and also form a matrix to support cell migration (46). For a number of reasons, the coagulation system as a whole has been recently gaining momentum as a promising target for the treatment of various cancers (47). The specific role of TF in CNS tumors makes it an attractive target for new therapies. Accordingly, FVIIa was recently conjugated to EF24, a synthetic curcumin analog, and used as a drug delivery molecule based on its high affinity for TF-expressing cells (48). This approach is exciting since TF is aberrantly expressed only on tumor vascular endothelial cells and on cancer malignant cells, therefore reducing the side effects commonly associated with current treatments. Further characterization of the mechanisms linking TF and Met may thus provide interesting new informations on the potential usefulness of targeting key elements of the hemostatic system for the treatment of MBs.

Funding

Natural Sciences and Engineering Research Council of Canada; Montreal Center for Experimental Therapeutics in Cancer/Fond de Recherche en Santé de Québec/Canadian institutes of Health Research Strategic Training program in Cancer Experimental Therapeutics (to M.P.).

Acknowledgements

We thank Claude Potvin for her help with immunohistochemistry.

Conflict of Interest Statement: None declared.

References

1. Wechsler-Reya,R. *et al*. (2001) The developmental biology of brain tumors. *Annu. Rev. Neurosci.*, **24**, 385–428.
2. Rossi,A. *et al*. (2008) Medulloblastoma: from molecular pathology to therapy. *Clin. Cancer Res.*, **14**, 971–976.
3. Gilbertson,R.J. (2004) Medulloblastoma: signalling a change in treatment. *Lancet Oncol.*, **5**, 209–218.
4. Ellison,D. (2002) Classifying the medulloblastoma: insights from morphology and molecular genetics. *Neuropathol. Appl. Neurobiol.*, **28**, 257–282.
5. Gilbertson,R.J. *et al*. (2008) The origins of medulloblastoma subtypes. *Annu. Rev. Pathol.*, **3**, 341–365.
6. Polkinghorn,W.R. *et al*. (2007) Medulloblastoma: tumorigenesis, current clinical paradigm, and efforts to improve risk stratification. *Nat. Clin. Pract. Oncol.*, **4**, 295–304.
7. Zwick,E. *et al*. (2002) Receptor tyrosine kinases as targets for anticancer drugs. *Trends Mol. Med.*, **8**, 17–23.
8. Goumnerova,L.C. (1996) Growth factor receptors and medulloblastoma. *J. Neurooncol.*, **29**, 85–89.
9. Grotzer,M.A. *et al*. (2000) Neurotrophin receptor TrkC predicts good clinical outcome in medulloblastoma and other primitive neuroectodermal brain tumors. *Klin. Padiatr.*, **212**, 196–199.
10. Duplan,S.M. *et al*. (2002) Antitumor activity of fibroblast growth factors (FGFs) for medulloblastoma may correlate with FGF receptor expression and tumor variant. *Clin. Cancer Res.*, **8**, 246–257.
11. Gilbertson,R.J. *et al*. (1998) Expression of the ErbB-neuregulin signaling network during human cerebellar development: implications for the biology of medulloblastoma. *Cancer Res.*, **58**, 3932–3941.
12. MacDonald,T.J. *et al*. (2001) Expression profiling of medulloblastoma: PDGFRA and the RAS/MAPK pathway as therapeutic targets for metastatic disease. *Nat. Genet.*, **29**, 143–152.
13. Gilbertson,R.J. *et al*. (2003) PDGFRB is overexpressed in metastatic medulloblastoma. *Nat. Genet.*, **35**, 197–198.
14. Hartmann,W. *et al*. (2005) Insulin-like growth factor II is involved in the proliferation control of medulloblastoma and its cerebellar precursor cells. *Am. J. Pathol.*, **166**, 1153–1162.
15. Birchmeier,C. *et al*. (2003) Met, metastasis, motility and more. *Nat. Rev. Mol. Cell Biol.*, **4**, 915–925.
16. Li,Y. *et al*. (2005) The scatter factor/hepatocyte growth factor: c-met pathway in human embryonal central nervous system tumor malignancy. *Cancer Res.*, **65**, 9355–9362.
17. Li,Y. *et al*. (2008) Functional and molecular interactions between the HGF/c-Met pathway and c-Myc in large-cell medulloblastoma. *Lab. Invest.*, **88**, 98–111.
18. Benvenuti,S. *et al*. (2007) The MET receptor tyrosine kinase in invasion and metastasis. *J. Cell. Physiol.*, **213**, 316–325.
19. Wang,X. *et al*. (2003) Potent and selective inhibitors of the Met [hepatocyte growth factor/scatter factor (HGF/SF) receptor] tyrosine kinase block HGF/SF-induced tumor cell growth and invasion. *Mol. Cancer Ther.*, **2**, 1085–1092.
20. Abounader,R. *et al*. (2005) Scatter factor/hepatocyte growth factor in brain tumor growth and angiogenesis. *Neuro Oncol.*, **7**, 436–451.
21. Hamasuna,R. *et al*. (1999) Regulation of matrix metalloproteinase-2 (MMP-2) by hepatocyte growth factor/scatter factor (HGF/SF) in human glioma cells: HGF/SF enhances MMP-2 expression and activation accompanying up-regulation of membrane type-1 MMP. *Int. J. Cancer*, **82**, 274–281.
22. Wang,H. *et al*. (2000) Hepatocyte growth factor enhances MMP activity in human endothelial cells. *Biochem. Biophys. Res. Commun.*, **272**, 900–905.
23. Moriyama,T. *et al*. (1998) Up-regulation of vascular endothelial growth factor induced by hepatocyte growth factor/scatter factor stimulation in human glioma cells. *Biochem. Biophys. Res. Commun.*, **249**, 73–77.
24. Boccaccio,C. *et al*. (2005) The MET oncogene drives a genetic programme linking cancer to haemostasis. *Nature*, **434**, 396–400.
25. Boccaccio,C. *et al*. (2005) A functional role for hemostasis in early cancer development. *Cancer Res.*, **65**, 8579–8582.
26. Wojtukiewicz,M.Z. *et al*. (2001) The hemostatic system and angiogenesis in malignancy. *Neoplasia*, **3**, 371–384.
27. Nijziel,M.R. *et al*. (2006) From Trousseau to angiogenesis: the link between the haemostatic system and cancer. *Neth. J. Med.*, **64**, 403–410.
28. Varki,A. (2007) Trousseau's syndrome: multiple definitions and multiple mechanisms. *Blood*, **110**, 1723–1729.
29. Price,G.C. *et al*. (2004) Tissue factor and tissue factor pathway inhibitor. *Anaesthesia*, **59**, 483–492.
30. Ott,I. *et al*. (2005) Tissue factor cytoplasmic domain stimulates migration by activation of the GTPase Rac1 and the mitogen-activated protein kinase p38. *Circulation*, **111**, 349–355.

31. Bromberg,M.E. *et al*. (1999) Role of tissue factor in metastasis: functions of the cytoplasmic and extracellular domains of the molecule. *Thromb. Haemost.*, **82**, 88–92.
32. Versteeg,H.H. (2004) Tissue factor as an evolutionary conserved cytokine receptor: implications for inflammation and signal transduction. *Semin. Hematol.*, **41**, 168–172.
33. Belting,M. *et al*. (2004) Regulation of angiogenesis by tissue factor cytoplasmic domain signaling. *Nat. Med.*, **10**, 502–509.
34. Dietzen,D.J. *et al*. (2004) Lipid rafts are necessary for tonic inhibition of cellular tissue factor procoagulant activity. *Blood*, **103**, 3038–3044.
35. Holen,T. *et al*. (2002) Positional effects of short interfering RNAs targeting the human coagulation trigger Tissue Factor. *Nucleic Acids Res.*, **30**, 1757–1766.
36. Provencal,M. *et al*. (2008) Tissue factor pathway inhibitor (TFPI) interferes with endothelial cell migration by inhibition of both the Erk pathway and focal adhesion proteins. *Thromb. Haemost.*, **99**, 576–585.
37. Fan,X. *et al*. (2003) hTERT gene amplification and increased mRNA expression in central nervous system embryonal tumors. *Am. J. Pathol.*, **162**, 1763–1769.
38. Guan,M. *et al*. (2002) Quantitative reverse transcription-PCR measurement of tissue factor mRNA in glioma. *Mol. Biotechnol.*, **20**, 123–129.
39. Bolanos-Garcia,V.M. (2005) MET meet adaptors: functional and structural implications in downstream signalling mediated by the Met receptor. *Mol. Cell. Biochem.*, **276**, 149–157.
40. Rong,Y. *et al*. (2005) PTEN and hypoxia regulate tissue factor expression and plasma coagulation by glioblastoma. *Cancer Res.*, **65**, 1406–1413.
41. Rong,Y. *et al*. (2006) Early growth response gene-1 regulates hypoxia-induced expression of tissue factor in glioblastoma multiforme through hypoxia-inducible factor-1-independent mechanisms. *Cancer Res.*, **66**, 7067–7074.
42. Takano,S. *et al*. (2000) Tissue factor, osteopontin, alphavbeta3 integrin expression in microvasculature of gliomas associated with vascular endothelial growth factor expression. *Br. J. Cancer*, **82**, 1967–1973.
43. Singh,V.P. *et al*. (2008) Src-mediated cortactin phosphorylation regulates actin localization and injurious blebbing in acinar cells. *Mol. Biol. Cell*, **19**, 2339–2347.
44. Herynk,M.H. *et al*. (2007) Activation of Src by c-Met overexpression mediates metastatic properties of colorectal carcinoma cells. *J. Exp. Ther. Oncol.*, **6**, 205–217.
45. Bardos,H. *et al*. (1996) Fibrin deposition in primary and metastatic human brain tumours. *Blood Coagul. Fibrinolysis*, **7**, 536–548.
46. Boccaccio,C. *et al*. (2006) Cancer and blood coagulation. *Cell. Mol. Life Sci.*, **63**, 1024–1027.
47. Ornstein,D.L. *et al*. (2002) The coagulation system as a target for the treatment of human gliomas. *Semin. Thromb. Hemost.*, **28**, 19–28.
48. Shoji,M. *et al*. (2008) Targeting tissue factor-expressing tumor angiogenesis and tumors with EF24 conjugated to factor VIIa. *J. Drug Target.*, **16**, 185–197.

Received November 17, 2008; revised March 25, 2009; accepted April 4, 2009