The Scatter Factor/Hepatocyte Growth Factor: c-Met Pathway in Human Embryonal Central Nervous System Tumor Malignancy

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Abstract
Embryonal central nervous system (CNS) tumors, which comprise medulloblastoma, are the most common malignant brain tumors in children. The role of the growth factor scatter factor/hepatocyte growth factor (SF/HGF) and its tyrosine kinase receptor c-Met in these tumors has been until now completely unknown. In the present study, we show that human embryonal CNS tumor cell lines and surgical tumor specimens express SF/HGF and c-Met. Furthermore, c-Met mRNA expression levels statistically significantly correlate with poor clinical outcome. Treatment of medulloblastoma cells with SF/HGF activates c-Met and downstream signal transduction as evidenced by c-Met, mitogen-activated protein kinase, and Akt phosphorylation. SF/HGF induces tumor cell proliferation, anchorage-independent growth, and cell cycle progression beyond the G1-S checkpoint. Using dominant-negative Cdk2 and a degradation stable p27 mutant, we show that cell cycle progression induced by SF/HGF requires Cdk2 function and p27 inhibition. SF/HGF also protects medulloblastoma cells against apoptosis induced by chemotherapy. This cytoprotective effect is associated with reduction of proapoptotic cleaved poly(ADP-ribose) polymerase and cleaved caspase-3 proteins and requires phosphoinositide 3-kinase activity. SF/HGF gene transfer to medulloblastoma cells strongly enhances the in vivo growth of s.c. and intracranial tumor xenografts. SF/HGF overexpressing medulloblastoma xenografts exhibit increased invasion and morphologic changes that resemble human large cell anaplastic medulloblastoma. This first characterization establishes SF/HGF:c-Met as a new pathway of malignancy with multifunctional effects in human embryonal CNS tumors. (Cancer Res 2005; 65(20): 9355-62)

Introduction
Embryonal central nervous system (CNS) tumors, known as medulloblastoma in the cerebellum and primitive neuroectodermal tumors (PNET) elsewhere in the CNS, are among the most frequent causes of cancer death in children. Despite aggressive therapy—consisting of surgery, radiation, and chemotherapy—lethality and morbidity of patients with embryonal CNS tumors remains high (1–3). Medulloblastoma is thought to arise from granule cell precursors in the external germinal layer of the developing cerebellum (4). They are highly invasive neoplasms with a tendency to disseminate throughout the CNS, leading to leptomeningeal metastasis (5). Understanding the molecular pathways involved in embryonal CNS tumor formation and growth is required for the identification of new therapeutic targets. Recent research on the molecular basis of these tumors and its implications for targeted therapeutics has focused on the Hedgehog-Patched, Wnt, and Trk signaling pathways (3, 4, 6). These pathways, whereas important, are recognized to represent a subset of the multiple molecular mediators of embryonal CNS tumorigenesis and malignancy. The SF/HGF:c-Met pathway has emerged as an important contributor to human neoplasia (7). However, the role of SF/HGF and c-Met in human embryonal CNS tumors is, to our best knowledge, completely unknown.

SF/HGF is a multifunctional growth factor that plays a role in the regulation of cell growth, cell motility, morphogenesis, and angiogenesis (8). SF/HGF exerts its effects through its only known receptor, the c-Met proto-oncogene product, a transmembrane receptor that possesses tyrosine kinase activity (9). SF/HGF and c-Met are expressed in a wide variety of human carcinomas where their overexpression or misexpression often correlates with poor prognosis (7). Activation of c-Met in tumors occurs through paracrine and autocrine mechanisms. Ligand-independent activation of c-Met has also been observed in tumor cells that express high levels of the receptor (10). Additionally, some types of cancers and metastatic lesions have activating mutations of c-Met (11, 12). We and others have shown that SF/HGF:c-Met affects tumorigenicity and malignant progression by inducing cell cycle progression, tumor cell migration, and tumor angiogenesis and by inhibiting apoptosis (13–17). The mitogenic effects of SF/HGF on tumor cells are at least partly due to its ability to mediate G1-S cell cycle transition (17). SF/HGF expression inhibits apoptosis of glioblastoma tumor cells and confers resistance to cell death induced by chemotheraphy and radiation (15, 16). Many studies have also shown that c-Met activation induces tumor cell migration and invasion (18, 19). c-Met is also a potent tumor angiogenic factor and its inhibition leads to inhibition of tumor growth partially via its effects on tumor angiogenesis (14, 15, 20, 21). SF/HGF:c-Met malignant functions are mediated by a network of signal transduction pathways and transcriptional events. Activation of c-Met results in the recruitment of scaffolding proteins, such as Gab1 and growth factor receptor binding protein 2, which lead to the activation of Ras and extracellular signal-regulated kinase/mitogen-activated protein kinase (MAPK). This causes changes in gene expression of cell cycle regulators, such as pRb, cdk6, and p27, as well as extracellular matrix proteinases, such as matrix metalloproteinases and urokinase-type plasminogen activator, leading to alterations of cytoskeletal and cell adhesive functions that control cell proliferation, migration, and invasion (7, 22, 23). Active c-Met also directly binds to phosphoinositide 3-kinase (PI3K) at phosphotyrosines 1,349 (Y-1349VHV) and 1,356 (Y-1356VNV; refs. 24, 25) of the receptor. This leads to the activation of Akt.
a well-characterized mediator of cell survival (26, 27). We and others have previously shown that activation of PI3K/Akt mediates SF/HGF-induced cancer cell resistance to apoptosis and cell death (16).

In the present study, we identify for the first time a role for the SF/HGF:c-Met pathway in human embryonal CNS tumor malignancy. We show that SF/HGF and c-Met expression levels in human tumor specimens significantly correlate with patient survival. SF/HGF activates c-Met-dependent signal transduction pathways in medulloblastoma cells, leading to induction of multiple malignancy parameters, including anchorage-independent growth, cell proliferation, cell cycle progression, and cell survival. Activation of c-Met in medulloblastoma cells also strongly induces in vivo medulloblastoma xenograft growth. These findings show that the SF/HGF-c-Met pathway is an important mediator of medulloblastoma malignancy. The results provide a timely validation of the SF/HGF-c-Met pathway as a target for the promising small molecular inhibitors of c-Met and anti-SF/HGF antibodies that are expected to enter clinical trials soon.

Materials and Methods

Cell culture and Methods. DAOY cells were grown in Improved MEM Zinc Option supplemented with 10% fetal bovine serum (FBS). D425 and D283 cells were grown in Improved MEM Zinc Option supplemented with 20% FBS. PESK and ONS-76 cells were grown in RPMI 1640 with L-glutamine supplemented with 10% FBS. All cells were grown at 37°C in 5% CO2-95% O2. For treatment with SF/HGF, cells were grown to ~50% confluence before being transfected to medium containing 0.1% FBS overnight. Cells were then incubated with 10 to 100 µg/mL recombinant SF/HGF (a kind gift from Genentech, Inc., San Francisco, CA) for the appropriate time points. For treatment with the MAPK inhibitor (PD-98059) and PI3K inhibitor (LY 294002 and wortmannin), cells were handled as described above except that they were preincubated with the inhibitor for 1 hour before being subjected to SF/HGF treatment. For treatment with camptothecin, cells were preincubated with SF/HGF for 48 hours before incubation with camptothecin for 48 hours. All five above-described cell lines were tested for SF/HGF and c-Met expression as well as for phosphorylation of c-Met, Akt, and MAPK in response to SF/HGF. DAOY and D425 and ONS-76 cells were tested for cell proliferation, cell cycle progression, and cytoprotection. DAOY and D425 cells were additionally tested for anchorage-independent growth as described below.

Vectors and transfections. All transfections were done using Fugene transfection reagent (Roche Biochemicals, Indianapolis, IN) according to the instructions of the manufacturer. For the generation of SF/HGF-overexpressing medulloblastoma cells, cDNA containing the complete 2.2 kb coding sequence for SF/HGF was subcloned into the pcDNA 3.1/Zeo mammalian expression vector (Invitrogen, Carlsbad, CA) to yield the pcDNA/pc vector. Empty pcDNA 3.1/Zeo vector was used for control transfections. Transfected cells were selected for zeocin resistance and all resistant clones were randomly pooled for experimental (DAOY-SF) and control (DAOY-Control) groups. This approach insures that all cells are stably transfected while avoiding the disadvantages associated with the use of single clones. SF/HGF expression levels were quantified by immunoblot analysis as described below. The dominant-negative form of Cdk2 (pCMV-Cdk2DN) was kindly provided by B. van den Heuvel (Massachusetts General Hospital, Charlestown, MA; ref. 28). The p27 degradation stabilizing mutant p27T187A construct was provided by S. van den Heuvel (Massachusetts General Hospital, Charlestown, MA). Blots hybridized with SF/HGF and cleaved PARP antibodies were stripped and reprobed with actin antibodies. Blots hybridized with phospho-SF/HGF and phosphoAkt antibodies were stripped and reprobed with total MAPK and Akt antibodies.

Clinical samples. Frozen tissues from 32 embryonal tumors resected between 1992 and 2002 at the Johns Hopkins Hospital were used to make the RNA to study the correlation between SF/HGF, c-Met, and patient survival. The cases included fourteen classic medulloblastomas, six anaplastic medulloblastomas, five nodular medulloblastomas, four supratentorial PNET, two medulloepitheliomas, and one pineoblastoma. The median age of patients was 8 years, and the median follow-up was 18 months. Eight additional human embryonal tumors were used for analysis of SF/HGF and c-Met protein by immunoblotting.

Quantitative reverse transcription-PCR. Total RNA was extracted from snap-frozen tumor tissues using TRIzol Reagent (Invitrogen) per the instructions of the manufacturer. RNA was then treated with DNase and further purified using the RNeasy Protocol (Qiagen, Valencia, CA). Quantitative reverse transcription-PCR (RT-PCR) was done using the iCycler (Bio-Rad Laboratories, Inc., Hercules, CA) with TaqMan One-Step RT-PCR Master Mix reagents (Applied Biosystems, Foster City, CA) and normalized to actin (Applied Biosystems) as previously described (30). Assays-on-Demand TaqMan probes (Applied Biosystems) were used to measure c-Met (assay ID: Hs00179845_m1) and HGF (assay ID: Hs00300159_m1) expression. All samples were analyzed in triplicate. Serial dilutions of total RNA from the medulloblastoma cell line DAOY (for c-Met) and astrocytoma cell line SW1088 (for HGF) were used to generate standard curves. The expression of c-Met and HGF in all samples was calculated in relation to these. The GraphPad Prism 4 program (San Diego, CA) was used for log-rank analysis of Kaplan-Meier survival curves.

Immunoblotting. Protein levels of c-Met and SF/HGF, p27, Cdk2, cyclin E, cyclin D1, E2F-1, caspase-3, cleaved caspase-3, cleaved poly(ADP-ribose) polymerase (PARP), and actin as well as phosphoprotein levels of c-Met, MAPK, and Akt in cells or tumor specimens were analyzed by immunoblotting. Immunoblotting was done as previously described using antibodies specific for SF/HGF, c-Met, Cdk2, cyclin E, cyclin D1, E2F-1 (Santa Cruz Biotechnologies, Santa Cruz, CA), p27 (BD Biosciences, San Jose, CA), MAPK, Akt, caspase-3, cleaved caspase-3, cleaved PARP phospho-c-Met, phospho-MAPK, and phospho-Akt (Cell Signaling Technologies, Beverly, MA; ref. 31). Blots hybridized with SF/HGF and cleaved PARP antibodies were stripped and reprobed with antiactin antibodies. Blots hybridized with phospho-MAPK and phospho-Akt antibodies were stripped and reprobed with total MAPK and Akt antibodies.

Cell proliferation and anchorage-independent colony formation in soft agar. Cell proliferation was assessed by seeding cells in medium containing 0.1% FBS (5,000/well) in 24-well plates. Cells were subsequently counted each day (n = 3) for 7 days using a Coulter counter and growth curves were established. Anchorage-independent growth was assessed by use of colony formation in soft agar as previously described (14). DAOY or D425 cells were suspended in soft agar (5,000 cells per well) and incubated at 37°C in 5% CO2-95% O2 in medium containing 1% FBS. Recombinant SF/HGF (10 ng/mL) was added to the experimental group twice per week; controls received medium only. After 3 weeks, the cells were fixed and stained with Wright’s solution. The number of colonies larger than 100 µm in diameter were determined by computer-assisted image analysis.

Propidium iodide flow cytometry. The cell cycle status was assessed using propidium iodide flow cytometry. Cells growing as a monolayer were trypsinized (Life Technologies, Grand Island, NY), pelleted by centrifugation, resuspended in 1 mL ice-cold PBS, and fixed by the addition of 4 mL ice-cold ethanol under gentle vortexing. Fixed cells were stored up to 1 week in 80% ethanol at −20°C until ready for labeling. Stored cells were collected by centrifugation, resuspended in 1 mL of PBS, and treated with 20 µg of DNase-free Rnase (Boehringer-Mannheim, Mannheim, Germany) for 30 minutes at 37°C. Cells were labeled with propidium iodide (Sigma, St. Louis, MO) at a final concentration of 100 µg/mL for 10 minutes at room temperature. Analyses were done on a FACScan (Becton Dickinson, Fullerton, CA). For experiments that did not involve transfection, 10,000 events were counted per sample. For experiments involving transfection, only GFP-labeled cells were counted (5,000 events per cycle). Because all vectors either coexpressed GFP or were cotransfected with GFP-expressing vectors, this ensured that only transfected cells were counted. Raw data...
were gated to remove doublets and cellular debris. The resultant cell cycle histograms were analyzed with CellQuest software (Becton Dickinson) and the area under the curve was integrated for each peak.

**Annexin V flow cytometry.** Apoptosis and cell death was assessed using Annexin V flow cytometry. To test the effect of SF/HGF on camptothecin-induced apoptosis, subconfluent proliferating cells were seeded in six-well plates and subsequently preincubated with or without HGF/SF (100 ng/ml) for 48 hours in 10% serum before treatment with camptothecin (20 μM/L for D225 cells and 0.5 μM/L for ONS-76 cells) for 48 hours. The fraction of phosphatidylserine-positive cells was determined by Annexin V labeling using the Annexin V-FITC Detection Kit according to the instructions of the manufacturer (BD PharMingen, San Diego, CA). Briefly, cells were pelleted and then resuspended in 100 μL binding buffer and 5 μL Annexin V-FITC and 10 μL propidium iodide were added, and the samples were incubated for 15 minutes at room temperature in the dark. The cell suspensions were diluted with 400 μL binding buffer and were immediately analyzed with flow cytometry as described above.

**Lactate dehydrogenase assays.** To further determine the effects of SF/ HGF treatment on cell viability, lactate dehydrogenase (LDH) assays were done using CytoTox96Nonradioactive Cytotoxicity Assay (Promega, Madison, WI). Cells were plated at 0.1 × 10⁶ per well in a 24-well plate. Cells were then preincubated with SF/HGF for 48 hours and subsequently treated with camptothecin as described above for 48 hours. Cells in each well were assayed for LDH release as follows: 50 μL supernatant aliquots were transferred to 96-well assay plates with 50 μL of reconstituted substrate mix and incubated at room temperature for 30 minutes in the dark. Stop solution (50 μL) was added to each well and the absorbance was read at 490 nm by an automatic spectrometer.

**Tumor formation in vivo.** The effects of c-Met activation on in vivo medulloblastoma xenograft growth were tested in s.c. and intracranial animal models. Due to slow tumor formation in s.c. xenografts, DAOY cells (5 × 10⁵ cells) were implanted into the flanks of Nu/Nu immunodeficient mice. For s.c. xenografts, DAOY-SF medulloblastoma cells (5 × 10⁵ cells) were implanted into the flanks of nude mice and animals were sacrificed at day 21. The brains were removed, sectioned, and stained with H&E. Maximal tumor cross-sectional area was measured by computer-assisted image analysis and tumor volumes were calculated.

**Statistical analysis.** Two-group comparisons were analyzed by two-sided Student’s t-test. Multiple-group comparisons were done with Bonferroni/Dunn multiple comparison tests. Survival data were analyzed with log-rank analysis of Kaplan-Meier survival curves. P values were determined for all analyses and P < 0.05 was considered significant.

**Figure 1.** Human medulloblastoma tumors and cells express c-Met and SF/HGF protein and high c-Met expression is associated with poor clinical outcome. A, cell extracts were isolated from embryonal CNS tumor surgical specimens and cells and analyzed for SF/HGF and c-Met protein by immunoblotting. All tumors and cell lines expressed both c-Met and SF/HGF. B, log-rank analysis of Kaplan-Meier survival curves for patients with above median (high Met) or below median (low Met) c-Met mRNA levels determined by real-time RT-PCR reveals significantly worse clinical outcomes for patients with high c-Met-expressing tumors.

**Results**

**Human medulloblastoma cells and tumors express c-Met and scatter factor/hepatocyte growth factor.** We analyzed SF/HGF and c-Met mRNA and protein expression in human embryonal CNS tumor cell lines and surgical tumor specimens by quantitative RT-PCR and immunoblotting. All five cell lines examined (DAOY, D425, D283, PFSK, and ONS-76) expressed moderate to high levels of c-Met and SF/HGF protein compared with the levels observed in glioma cells (Fig. 1A). All eight tumor specimens that were analyzed by immunoblotting expressed both c-Met and SF/HGF protein (Fig. 1A). All 32 additional embryonal CNS tumor specimens that were analyzed by RT-PCR were found to express c-Met mRNA and all but one tumor expressed SF/HGF mRNA (not shown). We also examined expression of c-Met protein in human medulloblastoma samples immunohistochemically. Consistent with the immunoblot data, all but one of 20 medulloblastomas examined contained cytoplasmic c-Met immunoreactivity (not shown). These data show that SF/HGF and c-Met are widely expressed in human embryonal CNS tumor specimens and cultured cells.

**c-Met messenger RNA expression is associated with poor clinical outcome.** We used real-time RT-PCR to measure the level of c-Met in mRNA extracted from snap-frozen tumor tissue. All embryonal CNS tumors examined (n = 32) contained detectable levels of c-Met mRNA. We divided these tumors into those with above and below median c-Met expression. Of the 16 tumors that exhibited higher than median expression levels of c-Met, 11 also had higher than median expression levels of SF/HGF. Log-rank analysis of Kaplan-Meier survival curves revealed that the high c-Met patient group had statistically significant shorter survival compared with low c-Met patients (P < 0.05; Fig. 1B). We also analyzed SF/HGF mRNA expression in this tumor set, and detected expression of the ligand in all but one of the cases examined. Whereas not statistically significant, there was a trend toward shorter survival in patients whose tumors contained above median levels of SF/HGF (P = 0.064; Fig. 1B).

**Scatter factor/hepatocyte growth factor activates c-Met and downstream signaling in medulloblastoma cells.** To assess the functionality of the c-Met receptor, we examined the effects of SF/HGF on the activation of c-Met and its downstream signaling by analyzing c-Met, MAPK, and Akt phosphorylation in response to...
SF/HGF treatment in DAOY, D425, D283, and ONS-76 medulloblastoma cells strongly induced c-Met, MAPK, and Akt phosphorylation in all four cell lines examined, indicating that the SF/HGF:c-Met pathway is functional and leads to the activation of c-Met–dependent signal transduction in human embryonal CNS cells (Fig. 2).

**Scatter factor/hepatocyte growth factor induces medulloblastoma cell proliferation and anchorage-independent growth.** SF/HGF induced proliferation in DAOY and ONS-76 but not in D425 cells. Whereas control DAOY cells did not grow under low serum (0.1%) conditions, addition of 10 ng/mL recombinant SF/HGF dramatically and significantly increased DAOY cell numbers relative to control (Fig. 3A). Anchorage-independent growth is a well-characterized in vitro correlate of malignancy. Using colony formation in soft agar assays, we found that SF/HGF strongly induces DAOY and D425 medulloblastoma cell anchorage-independent growth. SF/HGF significantly increased the number of formed colonies from 4.0 ± 2.0 to 37.0 ± 5.9 (n = 6; P < 0.05) in DAOY cells and from 12.2 ± 2.6 to 27.7 ± 6.7 (n = 6; P < 0.05) in D425 cells (Fig. 3B). These data show that SF/HGF induces embryonal CNS tumor cell proliferation and anchorage-independent growth.

**Scatter factor/hepatocyte growth factor induces cell cycle progression in medulloblastoma cells in a p27- and Cdk2-dependent manner.** Using flow cytometry, we found that SF/HGF allows DAOY and ONS-76 medulloblastoma cells but not D425 cells to escape G0-G1 arrest induced by serum withdrawal, indicating that SF/HGF can enhance medulloblastoma growth partly by promoting tumor cell proliferation (Fig. 4A). SF/HGF reduced the G1-G0 fraction from 66.6 ± 1.6% to 50.3 ± 1.7% (P < 0.05) in DAOY cells and from 71.7% to 63.2% (n = 2) in ONS-76 cells. Treatment of DAOY cells with SF/HGF reduced p27 and induced Cdk2, cyclin D1, cyclin E, and E2F-1 protein levels as measured by immunoblotting (Fig. 4B). Additionally, transfection of DAOY cells with the p27 degradation stable mutant p27T187A or a Cdk2 dominant-negative (Cdk2-DN) inhibited SF/HGF-mediated cell cycle progression. Whereas SF/HGF reduced the G1-G0 fraction from 56.0 ± 1.8 to 44.0 ± 1.0 (n = 3; P < 0.05) in control transfected cells, no reduction was measured in Cdk2-DN– and p27T187A-transfected cells (Fig. 4C). This shows that the SF/HGF-induced cell cycle response requires p27 degradation and Cdk2 function.

**Scatter factor/hepatocyte growth factor protects medulloblastoma cells against chemotherapy-induced cell death in a phosphoinositide 3-kinase–dependent manner.** We found that SF/HGF protects D425 and ONS-76 but not DAOY medulloblastoma cells against chemotherapy-induced cell death. Pretreatment of D425 or ONS-76 cells with SF/HGF before treatment with camptothecin reduced the fraction of dead cells from 52.5 ± 3.0% to 41.5 ± 1.4% (P < 0.05) in D425 cells and from 63.7 ± 2.0 to 46.7 ± 2.2 (P < 0.05) in ONS-76 cells as assessed by Annexin V flow cytometry (Fig. 5A). Cytoprotection was also confirmed by LDH

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**Figure 2.** SF/HGF activates c-Met and downstream signaling pathways in human medulloblastoma cells: DAOY, D425, D283, and ONS-76 medulloblastoma cells were treated with recombinant SF/HGF and analyzed for phospho-Met, phospho-MAPK, and phospho-Akt by immunoblotting. Blots were also hybridized to total MAPK and total Akt as control.

**Figure 3.** SF/HGF induces cell proliferation and anchorage-independent growth in medulloblastoma cells: A, SF/HGF induces the proliferation of DAOY and ONS-76 medulloblastoma cells. Cells (5,000 per well; n = 3 wells) were seeded in media containing 1% serum and treated with or without 10 ng/mL SF/HGF. Cells were counted and growth curves were established. B, assay of clonogenicity in soft agar showing that SF/HGF induces anchorage-independent growth. DAOY and D425 cells were suspended in soft agar and treated with 10 ng/mL SF/HGF for 3 weeks. Colonies >100 μm diameter were measured and counted by computer-assisted image. *P < 0.05.
assay (not shown). Basal apoptosis/necrosis levels were slightly but not statistically significantly inhibited by SF/HGF pretreatment of the cells. Consistent with the above findings, SF/HGF decreased the levels of the proapoptotic proteins cleaved PARP and cleaved caspase-3 in cells treated as described above (Fig. 5B). Pretreatment of D425 cells with the PI3K inhibitors LY 294002 or wortmannin, but not with the MAPK kinase inhibitor PD-98059, completely inhibited the cytoprotective effect of SF/HGF, showing that this effect is PI3K dependent (Fig. 5C).

Scatter factor/hepatocyte growth factor strongly enhances the growth of s.c. and intracranial medulloblastoma tumor xenografts. DAOY cells engineered to overexpress SF/HGF (DAOY-SF) or control cells expressing empty plasmids (DAOY-Control) were implanted s.c. in immunodeficient mice. DAOY-SF cells exhibited significantly faster tumor growth rates than tumors generated form DAOY-Control cells. Also, DAOY-SF xenograft growth rates were proportional to the SF/HGF expression levels of the transfected cells. DAOY-Control cells exhibited a very slow growth rate or did not grow at all (Fig. 6A).

DAOY-SF cells were stereotactically injected into the brains of immunodeficient mice. After 3 weeks, DAOY-SF xenografts grew significantly larger (maximal tumor cross-sectional area = 17.8 ± 2.9, n = 8) than xenografts established from DAOY-Control cells (maximal tumor cross sectional area = 0.64 ± 0.19, n = 9; Fig. 6B). DAOY-SF xenografts also showed more invasive characteristics with fingerlike protrusions, metastatic growth, and leptomeningeal spread as opposed to DAOY-Control xenografts that exhibited a more defined and limited growth (Fig. 6B).

When examined histologically, s.c. as well as intracranial DAOY-SF xenografts exhibited dramatic histologic differences compared with DAOY-Control xenografts. DAOY-SF xenografts displayed features that closely resemble those of human large cell anaplastic medulloblastoma (Fig. 6C). The large cell anaplastic medulloblastoma subtype is characterized by increases in cell size, mitotic rate, apoptotic rate, and prominent nucleoli. Xenografts from wild-type or vector-transfected DAOY cells were composed of round to spindled cells with vesicular chromatin, interspersed with occasional groups of larger cells with more prominent nucleoli. Single apoptotic cells were present, but clusters of apoptotic cells were generally not seen. Xenografts from DAOY-SF cells contained numerous enlarged cells, often with macronucleoli. Apoptotic bodies were common, as were large "apoptotic lakes" like those seen in human large cell anaplastic medulloblastoma. These tumors thus recapitulate several features of human large cell anaplastic medulloblastoma. Additionally, of the five anaplastic medulloblastomas among the human tumor specimens described above, four expressed higher than median levels of SF/HGF and two expressed higher than median levels of both SF/HGF and c-Met. These findings are consistent with a role of SF/HGF in generating this most malignant medulloblastoma subtype.

Discussion
This is, to the best of our knowledge, the first study that examines the role of the SF/HGF:c-Met pathway in human embryonal CNS tumor malignancy. The study shows a role for SF/HGF in embryonal CNS tumor growth and establishes the c-Met pathway as an interesting new potential target for experimental therapies.

The embryonal CNS tumor cell lines used in this study were carefully selected to represent various embryonal CNS tumor subtypes and disease models. The ONS-76 cell line was derived from a case of classic medulloblastoma and has also been used as an experimental model of leptomeningeal dissemination (32, 33). Using expression profiling, McDonald et al. (34) compared DAOY cells to medulloblastoma tumors and found them to be excellent
representatives of metastatic medulloblastoma. D283 and D425, two well-characterized medulloblastoma lines, are phenotypically different from each other as well as from DAOY (35, 36). The PFSK cell line is derived from a PNET tumor from the cerebral hemisphere and exhibits tumor stem cell characteristics (37). Also, all WHO types and subtypes of embryonal CNS tumors were represented in the human surgical tumor specimens used in the study. We found that c-Met and SF/HGF are expressed in all the above-mentioned human embryonal CNS tumor cells and surgical specimens. c-Met receptors were functional in all cell types examined as evidenced by the SF/HGF-induced phosphorylation of the receptor and associated downstream signaling molecules. This widespread expression and activation of c-Met is indicative of a critical role for the pathway in these tumors and is consistent with its expression and functional activity in cerebellar granule cells, which are believed to be the cell precursors for medulloblastoma (38). SF/HGF and c-Met were expressed in the same cells indicative of autocrine activation of the receptor. Autocrine loop formation has been associated with increased malignancy in other human tumors. Importantly also, c-Met expression levels in tumors significantly correlated with poor patient survival, indicating that c-Met levels could serve as a prognostic factor for medulloblastomas and providing the basis for subsequent mechanistic studies of the role of c-Met in promoting tumor growth.

The molecular mechanisms that govern medulloblastoma growth and malignancy are only partially understood. A few reports have shown an emerging role for the receptor tyrosine kinase pathways FGFR, PDGFR, Trk, and erbB in medulloblastoma (34, 39–41). However, the role of c-Met receptor tyrosine kinase in embryonal CNS tumors remained unexplored until now. Trks are the most studied receptor tyrosine kinases in medulloblastoma. A few studies have shown that TrkC receptor expression is associated with better prognosis of medulloblastoma patients and that activation of TrkC induces apoptosis of tumor cells (40, 42). Two reports also linked FGFR expression with antitumor activity in classic medulloblastoma subtype (39, 43). Therefore, unlike c-Met activation, which enhances embryonal CNS tumor growth and malignancy, activation of Trk and FGF receptors is associated with reduced tumor malignancy. Conversely, ERBB2 and PDGFR receptors have been linked to increased malignancy of medulloblastoma. Both ERBB2 and PDGFR expression in medulloblastoma have been associated with poor prognosis, metastatic phenotype, and enhanced medulloblastoma cell migration (34, 44, 45). Although our study did not systematically investigate tumor cell migration, DAOY-SF xenografts clearly displayed a more invasive phenotype than controls, indicating that SF/HGF also enhances embryonal CNS tumor migration and invasiveness in brain. We show a role for c-Met activation in regulating tumor cell cycle progression, proliferation, and survival mechanisms. Considering that SF/HGF is also a well-known and potent angiogenic factor, SF/HGF might, therefore, enhance medulloblastoma growth by acting on multiple malignancy parameters. This would explain the very
strong in vivo tumor-promoting effect of SF/HGF and render SF/HGF:c-Met a promising target for therapy.

Medulloblastoma tumors exhibit a high proliferative index. c-Met activation in medulloblastoma cells induced anchorage-independent growth, cell proliferation, and cell cycle progression. SF/HGF-induced cell cycle progression required inhibition of the tumor suppressor p27 as well as induction of Cdk2 and E2F-1. SF/HGF protected medulloblastoma cells against cell death and apoptosis induced by chemotherapy. This effect was mediated by PI3K. This cytoprotective effect indicates that targeting SF/HGF:c-Met could sensitize embryonal CNS tumors to chemotherapy and radiotherapy as already shown by our group in glioma (46). Therefore, the pathways that mediate SF/HGF effects on embryonal CNS tumors are similar to the ones observed in other human tumors, including gliomas. Of note is that SF/HGF did not affect all malignancy end points equally in all cell lines that were tested. In fact, SF/HGF induced cell proliferation and cell cycle progression but not cytoprotection in DAOY cells. Conversely, SF/HGF induced cytoprotection but not cell cycle progression and cell proliferation in D425 cells. SF/HGF affected all three above end points in ONS-76 cells. This heterogeneous response to SF/HGF might be due to the different origins of the cell lines and/or also reflect the cellular heterogeneity that is observed in embryonal CNS tumors.

SF/HGF gene transfer to DAOY medulloblastoma cells led to a strong induction of in vivo xenograft growth. SF/HGF also enhanced medulloblastoma tumorigenicity as evidenced by the transformation of DAOY from low tumorigenic in experimental animals to cells that form large and invasive xenografts. SF/HGF also changed the cell and tumor morphology. We found that SF/HGF-overexpressing DAOY xenografts exhibit dramatic histologic differences compared with control xenografts. DAOY-SF/HGF xenografts had enlarged cells with large nuclei and prominent nucleoli (Fig. 6). These features closely resemble the ones observed in human large cell anaplastic medulloblastoma, which possesses the worst prognosis among medulloblastoma subtypes. Overall, the tumor growth pattern was also changed by overexpression of SF/HGF. Unlike control xenografts, SF/HGF-overexpressing xenografts formed fingerlike protrusions and metastasis and invaded the subarachnoidal space. These characteristics are typical for

**Figure 6.** SF/HGF strongly enhances the growth of s.c. and intracranial medulloblastoma tumor xenografts and alters tumor morphology and histology: A, DAOY medulloblastoma cells were engineered to stably express full-length human SF/HGF. Stably transfected cells (mixed transfectants—not clones) were screened for SF/HGF protein levels by immunoblotting (right) and designated SF/HGF/A and SF/HGF/B. Control cultures (Control/A and Control/B) were transfected with empty vectors. All cultures were mixed with Matrigel (50% volume) and injected in the flanks of nude mice (5 × 10⁶ cells/animal, n = 9). Tumor growth was monitored by caliper measurements. The results show that tumor growth rates correlate with SF/HGF expression. B, SF/HGF-overexpressing and control-transfected DAOY medulloblastoma cells were stereotactically injected into the brains of immunodeficient mice. The animals (n = 10) were sacrificed after 4 weeks and maximum cross-sectional tumor areas were measured on H&E-stained brain cross sections. SF/HGF-overexpressing cultures generated significantly larger tumors than controls. SF/HGF transfectants were also more invasive and metastasized in the subarachnoidal space (arrows). C, histologic analysis of DAOY-SF xenografts showing enlarged cells with large nuclei and prominent nucleoli. These features closely resemble human large cell anaplastic medulloblastoma.
large-cell anaplastic human medulloblastomas. Therefore, c-Met activation in medulloblastoma might lead to the generation of this most aggressive medulloblastoma subtype. Large-cell anaplastic human medulloblastoma is also associated with c-Myc amplification or overexpression. We have previously shown that c-Myc induction by SF/HGF mediates G1-S transition in human glioma cells. However, a relationship between c-Met activation and c-Myc overexpression in large cell anaplastic medulloblastoma is presently undefined and is subject to ongoing investigation in our laboratory.

In conclusion, we have established for the first time a role for the SF/HGF-c-Met pathway in human embryonal CNS tumor malignancy. We showed that c-Met levels correlate with patient prognosis and that activation of the pathway has widespread and multifunctional tumor-promoting effects. Our findings provide preclinical evidence that the SF/HGF-c-Met pathway constitutes a promising new therapeutic target in embryonal CNS tumors and support testing of emerging c-Met pathway inhibitors, such as small molecule inhibitors and neutralizing anti-SF/HGF monoclonal antibodies, in these common childhood malignancies.

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