Expression of FoxM1 Is Required for the Proliferation of Medulloblastoma Cells and Indicates Worse Survival of Patients

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Abstract

Purpose: The transcription factor Forkhead box M1 (FoxM1) is a key regulator of cell-cycle progression. It is involved in the development of multiple organs, and we have previously reported on its important role for the mitotic entry of cerebellar granule neuron precursors. Constitutive expression of FoxM1 is required for the growth of multiple cancer types. This study aimed to determine its role in medulloblastoma, the most frequent malignant brain tumor in childhood that can derive from cerebellar granule neuron precursors.

Experimental Design: We evaluated the expression of FoxM1 together with its prognostic value in two independent series of human medulloblastoma samples using immunohistochemistry (n = 43) and gene expression arrays (n = 193). The functional impact of FoxM1 expression was characterized by knockdown experiments in four human medulloblastoma cell lines, and the thiazole antibiotic siomycin A was tested to downregulate FoxM1 and inhibit tumor cell growth.

Results: FoxM1 was highly expressed in all subtypes of medulloblastoma. Importantly, expression levels of FoxM1 significantly correlated with unfavorable clinical outcome in univariate analysis (P = 0.0005), and FoxM1 was identified as an independent prognostic marker by multivariate analysis (P = 0.037). Knockdown of FoxM1 in medulloblastoma cell lines resulted in a significant decrease of cell viability which was caused by a failure in mitotic spindle formation and caspase-dependent mitotic catastrophe. Siomycin A significantly inhibited the expression of FoxM1 and the growth of medulloblastoma cells.

Conclusions: FoxM1 may be used as an additional prognostic marker and may represent a potential novel target to treat patients suffering from medulloblastoma.

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Introduction

Medulloblastoma is the most common malignant brain tumor in childhood with a 5-year overall survival rate of 66% (1). State-of-the-art treatment combines surgery, craniospinal irradiation, and multiple chemotherapeutics with severe sequelae of toxic side effects (2–4). The development of novel treatment options is therefore urgently needed. Medulloblastoma is a heterogeneous disease with 6 subgroups that differ with respect to histology, molecular expression profiles, genetics, and clinical outcome (5). One of these subgroups is associated with a constitutive activation of the Sonic hedgehog signaling pathway, which may, for instance, be caused by mutations of genes encoding the Hedgehog receptors Patched or Smoothened (6, 7). While the cellular origin of medulloblastoma is uncertain for most of the 6 subgroups, tumors associated with pathologic Hedgehog signaling have recently been shown to arise from cerebellar granule neuron precursors (8). During normal development, these precursors extensively proliferate due to a physiologic stimulation by Sonic hedgehog (9) until they migrate away from their mitotic niche in the external granule cell layer and differentiate at postnatal stages (10). While the mechanisms of differentiation in cerebellar granule neurons are not yet clearly understood, it is evident that their cell-cycle progression is dependent on FoxM1 (11), which is a target gene of Hedgehog signaling (12, 13).

FoxM1 (previously known as HFH-11B, MPP2, or Trident) is a member of the Forkhead family of transcription factors that share homology within the winged-helix/Forkhead DNA-binding domain (14, 15). FoxM1 is predominantly expressed in fetal tissues, but its expression may be maintained in proliferating adult tissues (15, 16). FoxM1 is a key regulator of the transition from G1 to S-phase by activating genes required for cell-cycle progression such as...
Translational Relevance

Medulloblastoma is the most common malignant brain tumor in childhood. It is related to unsatisfactory survival rates, and the development of causal therapies is highly desired. We show here that expression of the transcription factor FoxM1 may serve as an independent prognostic marker which easily allows clinicians to individually adjust therapy strategies. We further show that FoxM1 is essential for the growth of medulloblastoma cells and may serve as therapeutic target. Last, the antibiotic siomycin A proved to successfully downregulate FoxM1 and to inhibit tumor cell growth. Our studies may therefore serve as a basis for clinical trials including expression of FoxM1 as a prognostic marker to stratify patients and siomycin A as a novel drug to target medulloblastoma.

Materials and Methods

Tissue samples and cell lines

A total of 43 formalin-fixed, paraffin-embedded surgical tumor samples from patients with medulloblastoma were analyzed. Inclusion of patients in the study was unbiased and only dependent on the availability of sufficient tumor material and clinical follow-up data. Patients included 21 males (48.8%) and 22 females (51.2%). They were treated in the University Hospitals of Munich, Göttingen, Bremen, Hannover, and Münster (all Germany). Nineteen children were treated in prospective multicenter trials as described: (i) HIT 2000 trial (n = 9; stratification of risk-adapted treatment according to age and metastasis stage into 4 different treatment arms with combination of postoperative chemotherapy and/or radiotherapy and/or maintenance chemotherapy), (ii) HIT’91 trial (n = 6; randomized trial of postoperative chemotherapy followed by craniospinal radiotherapy vs. postoperative craniospinal radiotherapy followed by maintenance chemotherapy; ref. 24), (iii) HIT-MED’99 (n = 2; stratification of risk-adapted treatment according to age and metastasis stage into 4 different treatment arms), (iv) HIT’88 trial (n = 1; preradiation chemotherapy and radiotherapy; ref. 25), and (v) HIT’87 trial (n = 1; systemic interval chemotherapy until craniospinal radiotherapy was applied at 3 years of age or at relapse; ref. 26). The median age was 9.3 years (range, 0.6–45.6 years). The median follow-up of survivors was 53 months (range, 22.2–175.6 months). Sixteen patients succumbed to their disease, 25 were alive as of January 1, 2011. The study included 1 anaplastic medulloblastoma, 25 medulloblastomas of classical histology, 14 medulloblastomas of desmoplastic histology, and 2 medulloblastomas with extensive nodularity. One tumor was not classified. Detailed patient characteristics are given in Supplementary Table S1. Tumor diagnosis was established by standard light microscopic evaluation of hematoxylin and eosin–stained sections and silver stains. Diagnoses were made independently by at least 2 neuropathologists, based on the criteria of the latest WHO brain tumor classification (27). The human medulloblastoma cell line DAOY as well as HEK293T cells were purchased from the American Type Culture Collection. The human medulloblastoma cell line D425med was obtained from Dr. Darell Bigner (Duke University Medical Center, Durham, NC). The R300 and the UW228 human medulloblastoma cell lines were obtained from Dr. Michael S. Bobola (Children’s Hospital and Regional Medical Center, Seattle, WA).

Immunohistochemistry

Paraffin-embedded tissue was sectioned, deparaffinized, and rehydrated before heat-induced antigen retrieval was conducted. Immunohistochemical staining was done using primary antibodies against FoxM1 (1:25; Santa Cruz Biotechnology) and the HRP/DAB Staining System (DAKO) according to the manufacturer’s specifications. Hemalun was used for nuclear counterstaining. For immunofluorescent stainings, sections and fixed medulloblastoma cells from cell cultures were washed twice with PBS/0.1% Triton X-100 and then incubated in blocking buffer (1-Block protein-based blocking reagent; Applied Biosystems) for 30 minutes. Primary antibodies (FoxM1: 1:25, Santa Cruz Biotechnology; Ki67: 1:500, Dako; and cyclin B1: 1:200, Santa Cruz Biotechnology) were diluted in blocking buffer and applied over night at 4°C. Next, cells were washed twice with PBS/0.1% Triton X-100 and incubated for another 30 minutes with a 1:500 dilution of fluorescent-labeled secondary antibodies (Invitrogen) in blocking buffer. Cells

Cdc25B, cyclin B1, Aurora B kinase, and Polo-like kinase 1 (Plk1; refs. 14, 17). FoxM1 is also essential for chromosome segregation (16, 18), and depletion of FoxM1 leads to a failure to enter S-phase and impairs proper M phase completion with subsequent mitotic catastrophe (15, 17, 19). Increased levels of FoxM1 expression have been detected in many different types of human cancer such as basal cell carcinomas, breast cancer, glioblastomas, lung, pancreatic, and prostate cancer (13, 19–22). Within this context, FoxM1 has not only been identified as a prognostic marker (22), but its expression has also been shown to be required for the growth of lung cancer (20), prostate carcinomas (21), and breast cancer (19).

Previous work has shown a crucial role for FoxM1 during the development of cerebellar granule neuron precursors as well as a pathologically maintained expression of FoxM1 in tumors arisen in ptc−/− mice, a well-established mouse model for Sonic hedgehog–associated medulloblastoma (11, 23). However, the relevance of FoxM1 in human medulloblastoma has not yet been investigated. We show here that FoxM1 is highly expressed in human medulloblastomas and that it significantly correlates with patient’s outcome. Blocking of FoxM1 in medulloblastoma cell lines by RNA interference technology as well as by the chemical antibiotic siomycin A proved to successfully downregulate FoxM1 and to inhibit tumor cell growth. Our studies may therefore serve as a basis for clinical trials including expression of FoxM1 as a prognostic marker to stratify patients and siomycin A as a novel drug to target medulloblastoma.
were washed twice with PBS/0.1% Triton X-100, counter-
stained with 4',6-diamidino-2-phenylindole (DAPI), and
mounted in Immu-Mount (Thermo Shandon). All images
of tumor tissue were collected on an Olympus IX50 micro-
scope. Images from cell culture experiments were taken
using a confocal laser scanning microscope (Zeiss).

Cell culture
All cell lines were grown in Dulbecco’s Modified Eagle’s
Media containing 10% fetal calf serum and 1% glutamine at
37°C and 5% CO2 in a humidified atmosphere. Lentiviral
particles for cell line transductions were produced by trans-
fecting HEK293T cells with plasmids containing sequences
encoding for packaging and envelope proteins as well as for
short hairpin RNAs (shRNA) using FuGENE6 (Roche). The
plKO.1 vectors, including FOXM1 shRNA, as well as mock
sequences were provided by the Broad Institute of Harvard
and MIT (Cambridge, MA). shRNA sequences were GCAC-
TATACAAATAGCCCTAT (#1), GCCAATCGTTCTCTGACAG-
GAA (#2), and CCGCAGGTATGCACGCGT (mock). Super-
natant medium from transfected HEK293 cells including
viral particles was harvested 48 hours after transfection,
filtered, and stored at −80°C until use. Medulloblastoma
cells were transduced with viruses overnight before cells
were selected for those with successfully integrated shRNA
sequences by treatment with 2 μg/μl puromycin for 48
hours. Cell viability was measured using MTT assays accord-
ing to manufacturer’s instructions (Roche). For analysis of
the spindle apparatus, DAOY medulloblastoma cells were
transiently transfected with an α-tubulin-green fluorescent
protein (GFP) construct using the Nanolucie Transfection
Reagent Kit (Merck) before knockdown was conducted.
Finally, cells were seeded on glass-bottom plates (Mattek
Corporation) and incubated in a heated chamber at 37°C and
5% CO2 in a humidified atmosphere for 16 hours. Spindle
morphology of the living cells was visualized with a
confocal laser scanning microscope (Zeiss). The percentage
of cells with mitotic spindle defects was determined by
counting at least 200 mitotic cells from 3 different transsec-
tions for both negative control and FoxM1 shRNAs or
cell line transduced with siomycin A. Mitotic cells
with a regular bipolar spindle were defined as normal. Cells
were scored as abnormal if they displayed more than 2
spindle poles. Siomycin A was obtained from the NCI-
Chemotheerapeutic Agents Repository, stored in dimethyl
sulfoxide (DMSO), and applied to the cells for 48 hours
prior to protein extraction or MTT assays.

Western blotting
Standard semidy Western blotting was carried out as
described (11). Twenty-five micrograms of each protein
sample was separated on 10% SDS-PAGE and then trans-
ferred to Immobilon polyvinylidene difluoride membranes
(Millipore). The primary antibodies used were anti-FoxM1
(1:500; Santa Cruz Biotechnology), anti-cleaved caspase 3
(1:1,000; Cell Signaling Technology), anti-cyclin B1 (1:500;
Santa Cruz Biotechnology), anti-β-actin (1:5,000; Sigma),
and anti-β-tubulin (1:5,000; Sigma). Peroxidase-conjugat-
ed secondary antibodies included goat anti-mouse
(1:2,000; Jackson ImmunoResearch Laboratories) and goat
anti-rabbit (1:2,000; Pierce Biotechnology) antibodies. Blots
were developed using enhanced chemiluminescence
(Roche), according to the manufacturer’s instructions.
Signals were quantified using the ImageJ Software (NIH,
Bethesda, MD).

Statistical analysis
For analysis of the expression data and cell viabilities, the
Mann–Whitney U test was used to compare the median of 2
groups. To calculate the fraction of tumor cells labeled with
antibodies against FoxM1 and Ki67, at least 500 tumor cells
were counted for each tumor. Correlation of 2 paired data
sets was done with the Spearman correlation. Fractions
of mitotic cells with abnormal spindles in different experi-
mental conditions were compared using the Fisher exact
test. The Kaplan–Meier method was used to estimate sur-
vival, and log-rank tests were used to compare survival
between the respective groups. For all tests, a value of P <
0.05 was considered significant. Analyses were conducted
using Prism 5 (GraphPad) except for multivariate analyses
with Cox regression models that were done using PASW
statistics 18 (IBM).

Results
Expression pattern of FoxM1 in human
medulloblastoma tumor samples
We have previously conducted in situ hybridizations to
show expression of FoxM1 in murine cerebellar tissue (11).
In particular, we found that FoxM1 is expressed in granule
cell precursors of the developing cerebellum as well as in
medulloblastoma that arise in cell precursors of the developing
cerebellum as well as in medulloblastoma that arise in

cerebellar tissue. To see to what extent FOXM1 is expressed
in human medulloblastoma samples, we first reanalyzed
recently published global gene expression arrays of 193
ded in Supplementary Table S1. Expression of FoxM1
medulloblastoma tissue samples (5). As shown in Fig.
1A, medulloblastomas express high levels of FOXM1 with-
out significant differences between the distinct molecular
subgroups \( P = 0.0536 \). With respect to the different
histologic subtypes, FOXM1 is similarly expressed in classic
and nodular/desmoplastic medulloblastomas. However,
large cell/anaplastic medulloblastomas, which are charac-
tered by CMYC or NMYC amplification and worse survival
(28), express higher levels of FOXM1 than other histologic
subtypes \( P < 0.001 \; \text{[Fig. 1B]} \). Patients’ age did not correlate
with the expression of FOXM1 \( P = 0.5785 \; \text{[Supplementary
Fig. S1A]} \). Next, we used antibodies raised against FoxM1 on
sections from formalin-fixed, paraffin-embedded tumor
material of 43 medulloblastoma cases to visualize expres-
sion of FoxM1 in a cellular level. Histopathologic charac-
teristics of the tumors and patients’ clinical data are pro-
vided in Supplementary Table S1. Expression of FoxM1
protein was detectable in all analyzed medulloblastomas
and was restricted to the tumor cell nuclei (Fig. 1C). Tumors
displayed a median expression of 6.95%, ranging between
2.44% and 30.7% of nuclei binding antibodies against
As shown in Fig. 1C, FoxM1 expression in desmoplastic medulloblastomas was predominantly found in internodular tumor areas, which are highly proliferative, but only to a lesser extent in nodules that are usually more differentiated. We therefore asked whether FoxM1 might be expressed only in Ki67$^+$ tumor cells or whether expression of FoxM1 correlates with expression of Ki67 in medulloblastoma. As revealed by double immunofluorescent stainings, antibodies against FoxM1 may also bind to Ki67$^/$C0 tumor cells (Fig. 1E, arrowheads), and overall expression of FoxM1 did not correlate with the expression of Ki67 in sections of human medulloblastoma tissue samples ($n = 43$, $r_s = 0.254; P = 0.197$). Similarly to the data generated from expression arrays, we did not find significant differences of FoxM1 expression in classic and nodular/desmoplastic medulloblastoma ($P = 0.64$; Supplementary Fig. S1B). Also, FoxM1 protein expression was not dependent on the age of the patient ($P = 0.4082$; Supplementary Fig. S1C).

To test the clinical relevance of FoxM1 expression levels in medulloblastomas, we analyzed the survival of patients according to the protein expression of FoxM1 in medulloblastoma tumors by using the Kaplan–Meier method. Clinical follow-up data were available for 41 patients, and patients were dichotomized into 2 equally large groups using the median FoxM1 expression (7.02%) as cutoff point. The overall survival of patients with a high number of FoxM1$^+$ tumor cells ($n = 20$) was significantly worse than the survival of patients with a low number of FoxM1$^+$ tumor cells ($n = 21$; $P = 0.0005$; Fig. 2A). The 5-year overall survival rate was better in patients with medulloblastoma having low FoxM1-expressing tumors than in patients with...
high FoxM1-expressing tumors (FoxM1$_{\text{high}}$ group, 84.8% vs. FoxM1$_{\text{low}}$ group, 42.8%). To confirm these results in an independent cohort, we reanalyzed available microarray data from 130 patients with medulloblastoma and documented clinical follow-up (5). Here, patients were sorted on the basis of expression of FoxM1 and subsequently dichotomized on the basis of a series of FoxM1 expression levels. Log-rank significance was calculated for each cutoff value, and the best P value out of the sequence was used for further analysis. As shown in Fig. 2B, patients suffering from tumors with high levels of FoxM1 ($n = 44$) had a significantly worse outcome than patients with tumors that displayed only low levels of FoxM1 ($n = 86$; log-rank test, $P = 0.0055$). The same cohort of patients was originally used to identify a subgroup of patients with a distinct genetic fingerprint that drives poor clinical outcome (5). This "c1" subgroup is characterized genetically by $CMYC$ copy number gains and transcriptionally by enrichment of photoreceptor pathways and increased miR-183,96,182 expression. Further parameters that were previously found to predict the outcome of patients with medulloblastoma included Wnt pathway activation (29), large cell/anaplastic histology (28), and the presence of metastases (4). To evaluate whether the prognostic value of FoxM1 is independent of these characteristics, we conducted uni- and multivariate analyses using a Cox regression model. As

![Figure 2. Prognostic impact of FoxM1 expression in medulloblastoma. A, median fraction of FoxM1$^+$ tumor cells was set as threshold to divide patients into FoxM1$_{\text{high}}$ (n = 20) and FoxM1$_{\text{low}}$ (n = 21). Patients with tumors expressing low levels of FoxM1 protein survived significantly longer than patients with tumors expressing high levels of FoxM1 protein ($P = 0.0005$). B, survival rate analysis of a separate patients' cohort ($n = 130$) with FoxM1 expression analyzed by microarrays. Patients were sorted on the basis of expression of FoxM1 and subsequently dichotomized on the basis of FoxM1 expression. For group separation, the log-rank significance was calculated. The best P value out of the sequence was used to represent the final FoxM1 expression cutoff. High expression of FoxM1 ($n = 44$) was related to a significantly worse outcome than low expression of FoxM1 ($n = 86, P = 0.0055$). CI, confidence interval.](image)

### Table 1. Univariate Cox regression analysis and multivariate survival analysis of the microarray data set ($n = 130$)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>HR</th>
<th>95% CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Univariate survival analysis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FOXM1$<em>{\text{high}}$ expression (reference: FOXM1$</em>{\text{low}}$ expression)</td>
<td>2.67</td>
<td>1.30–5.49</td>
<td>0.008</td>
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<tr>
<td>LC/A Histology (reference: all other)</td>
<td>3.0</td>
<td>1.32–6.78</td>
<td>0.008</td>
</tr>
<tr>
<td>Molecular subgroup, MYC (c1) (reference: all other)</td>
<td>2.96</td>
<td>1.30–6.70</td>
<td>0.009</td>
</tr>
<tr>
<td>M stage, M+ (reference: M0)</td>
<td>1.73</td>
<td>0.70–4.26</td>
<td>0.235</td>
</tr>
<tr>
<td>Molecular subgroup, WNT (c6) (reference: all other)</td>
<td>0.35</td>
<td>0.05–2.54</td>
<td>0.295</td>
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<tr>
<td>B. Multivariate survival analysis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FOXM1$<em>{\text{high}}$ expression (reference: FOXM1$</em>{\text{low}}$ expression)</td>
<td>2.24</td>
<td>1.05–4.77</td>
<td>0.037</td>
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<tr>
<td>Molecular subgroup, MYC (c1) (reference: all other)</td>
<td>2.07</td>
<td>0.89–4.83</td>
<td>0.093</td>
</tr>
<tr>
<td>Molecular subgroup, WNT (c6) (reference: all other)</td>
<td>0.32</td>
<td>0.04–2.48</td>
<td>0.278</td>
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<tr>
<td>LC/A Histology (reference: all other)</td>
<td>1.56</td>
<td>0.63–3.86</td>
<td>0.332</td>
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<tr>
<td>M stage, M+ (reference: M0)</td>
<td>1.33</td>
<td>0.52–3.35</td>
<td>0.553</td>
</tr>
</tbody>
</table>

NOTE: Univariate Cox regression analysis of the microarray data set ($n = 130$) revealed that FoxM1 expression, LC/A histology, and c1 subgroup are significantly correlated with worse clinical outcome. Multivariate survival analysis of the microarray data set ($n = 130$) showed that FoxM1 expression was an independent prognostic factor ($P = 0.037$) with regard to molecular subgroups, histology, and M stage.

Abbreviation: CI, confidence interval.
shown by univariate Cox regression analyses, expression of FOXM1 again turned out as a prognostic marker in patients with medulloblastoma (\(P = 0.008\)), similar to large cell/anaplastic histology (\(P = 0.008\)) and a "c1" molecular profile (\(p = 0.009\); Table 1, univariate survival analysis). Although Wnt activation and metastatic disease did not reach statistical significance in univariate analyses of this series (Table 1, univariate survival analysis), they were included into the multivariate model to obtain estimates of HRs adjusted for them (Table 1, multivariate survival analysis). Using this multivariate model, we found that expression of FOXM1 is an independent prognostic marker for patients with medulloblastoma (HR, 2.24; 95% confidence interval, 1.05–4.77; \(P = 0.037\); Table 1, multivariate survival analysis).

Knockdown of FoxM1 reduces medulloblastoma cell growth by induction of mitotic catastrophe

To unravel the functional impact of FoxM1 in medulloblastoma, we aimed to knockdown FOXM1 expression in 4 permanent human medulloblastoma cell lines (D425med, DAOY, R300, and UW228) and measured cell viability in control and in knockdown conditions. As shown in Fig. 3A, all medulloblastoma cell lines expressed high levels of FoxM1 protein when transduced with lentiviral particles containing mock sequences. However, transduction with lentiviral particles containing 2 different shRNA sequences against FOXM1 resulted in a dramatic decrease in FoxM1 protein expression, as measured by Western blot analysis (Fig. 3A). Remaining levels of FoxM1 expression ranged between 9.23% and 47.66% compared with protein levels of mock transduced cells. To investigate whether high expression of FoxM1 might be a general phenomenon associated with proliferating cells, we further analyzed FoxM1 expression in HEK293T cells and found that this cell line expresses only very low levels of FoxM1 (Fig. 3A).

Cell viability was measured using MTT assays. Knockdown of FoxM1 decreased medulloblastoma cell viability significantly (up to 35% of mock-infected cells; \(P < 0.05\) for all medulloblastoma cell lines) in all 4 cell lines using both standard...
constructs (Fig. 3B). Interestingly, knockdown of FoxM1 in HEK293T cells did not affect cell viability significantly (Fig. 3B). Mechanistically, Western blot analysis revealed that cleaved caspase 3 was upregulated in all FoxM1-depleted medulloblastoma cell lines but not in HEK293T cells with a knockdown of FoxM1 (Fig. 3C). Upregulation of caspase 3 may occur as a part of mitotic catastrophe (30) which had previously been described both for FoxM1-deficient cerebellar granule neurons (11) and for tumor cells with a knockdown of FoxM1 (19). We therefore examined possible structural changes in cell spindle dynamics in dependence of FoxM1 expression. We visualized the spindle of DAOY medulloblastoma cells by transfecting cells with an α-tubulin-GFP construct (Fig. 4A). Transduced cells were seeded on glass-bottom plates and imaged 16 hours after transduction with knockdown constructs using a confocal laser scanning microscope. We observed significantly more mitotic cells with an abnormal spindle (57.2% and 63.4%), when FoxM1 is depleted by 2 different knockdown constructs (shRNA #1 and shRNA #2) compared with mock-infected cells (21.2%) or untreated cells (no virus; 3.2%; Fig. 4B; \( P < 0.05 \)). We further looked at the expression of cyclin B1 in such cells because enhanced expression of cyclin B1 as well as its translocation to the cell nucleus are indicators of a possible G2–M arrest of the cells (31, 32). As shown in Fig. 4C, knockdown of FoxM1 resulted in a shift of cyclin B1 to the nuclei of medulloblastoma cells, and subsequent Western blot analyses revealed that overall expression of cyclin B1 was clearly upregulated in FoxM1-deficient cells (Fig. 4D). Together, we suggest that knockdown of FoxM1 in medulloblastoma cells results in mitotic catastrophe as shown by enhanced expression of cleaved caspase 3, nuclear translocation of cyclin B1, and abnormal cell spindles.

Figure 4. Knockdown of FoxM1 induced mitotic spindle catastrophe. A, the medulloblastoma cell line DAOY was stably transfected with an α-tubulin-GFP (α-TUB-GFP) construct and seeded on glass-bottom plates (Mattek Corporation). Morphology of the spindle was visualized with a confocal laser scanning microscope (Zeiss) in wild-type (no virus), mock infected (mock virus), and FoxM1 siRNA–infected (shRNA #1 and shRNA #2) cells. Scale bar for all images, 10 \( \mu \)m. B, quantification of mitotic cells showed a significant \( P < 0.05 \) increase in cells with an abnormal spindle when FoxM1 was depleted with siRNA. *, \( P < 0.05 \). C, immunofluorescent stainings of DAOY medulloblastoma cell line for cyclin B1 (green) and DAPI. Cells showed nuclear localization of cyclin B1 when FoxM1 was downregulated by shRNAs. Scale bar, 20 \( \mu \)m. D, Western blot analysis shows increased expression of cyclin B1 when FoxM1 was downregulated. Numbers indicate level of cyclin B1 compared with control (mock virus). β-Actin was used as loading control.
Siomycin A reduces expression levels of FoxM1 and recapitulates effects of FoxM1 knockdown in medulloblastoma cells

The antibiotic thiazole compound siomycin A has previously been reported to represent a specific inhibitor of FoxM1 (33). We therefore tested whether siomycin A was able to reduce levels of FoxM1 in medulloblastoma cells and hence to inhibit tumor cell growth. As shown in Fig. 5A, treatment of human medulloblastoma cell lines D425med, DAOY, R300, and UW228 with siomycin A resulted in a decrease of FoxM1 protein levels in all human medulloblastoma cell lines and in HEK293T cells. Treatment with 10 µmol/L siomycin A resulted in a downregulation of FoxM1 protein levels to 48.20% (mean) in medulloblastoma and HEK293T cells (Fig. 5A). Next, cell viability was determined using MTT assays and showed a dose-dependent siomycin A–induced reduction of cell numbers in all treated human medulloblastoma cell lines (Fig. 5B). Interestingly, growth of HEK293T was not significantly affected by siomycin A, suggesting that siomycin A is not generally antiproliferative or toxic (Fig. 5B). High concentrations of siomycin A reduced cell viability below initial values (ΔOD < 0) in medulloblastoma cell lines, indicating a reduction of cells. We therefore assessed the expression of cleaved caspase 3 to determine whether programmed cell death or mitotic catastrophe would play a role in this context, similar to the situation observed for lentiviral knockdown of FOXM1 (Fig. 3). As shown by Western blot analyses shown in Fig. 5C, siomycin A induced upregulation of cleaved caspase 3 in medulloblastoma cells but not in HEK293T cells.

Figure 5. Treatment of human medulloblastoma cell lines with siomycin A. A, human medulloblastoma cell lines D425med, DAOY, R300, and UW228 as well as HEK293T cells were treated with 2 concentrations of siomycin A (Sio; 5 µmol/L and 10 µmol/L), and resulting protein levels were measured and compared with DMSO control (control). β-Tubulin was used as loading control. B, cell viability was decreased dose dependently with increasing concentrations of siomycin A in all medulloblastoma cell lines but not in HEK293T cells. C, expression of cleaved caspase 3 (Casp-3 cleaved) was induced when FoxM1 was downregulated in medulloblastoma cell lines but not in HEK293T cells.
HEK293T cells. Next, we analyzed the spindle formation in living tumor cells that were treated with siomycin A. α-Tubulin-GFP-transfected DAOY cells were seeded on glass-bottom plates and incubated in a heated chamber for 16 hours. Untreated cells, cells treated with DMSO, and cells treated with 5 μmol/L siomycin A were then imaged with a confocal laser scanning microscope. We observed a significant increase of mitotic cells with an abnormal spindle apparatus in siomycin A–treated cells (32.6%) as compared with DMSO-treated cells (3.4%, P = 0.049, Fig. 6A and B). No significant difference was observed with respect to the number of abnormal spindles in cells with DMSO treatment and cells without any treatment. To determine whether these findings may have resulted from mitotic catastrophe of the cells, we again looked for a possible G2–M arrest of the cells. Similar to the cells with a knockdown of FoxM1 (Fig. 4), cells treated with siomycin A showed a translocation of cyclin B1 to the nucleus (Fig. 6C) and an overexpression of cyclin B1 as revealed by Western blot analyses from siomycin A–treated tumor cells (Fig. 6D). Taken together, our data suggest that siomycin A is able to significantly down-regulate expression of FoxM1 and inhibits the growth of medulloblastoma cells.

Discussion

We report here that FoxM1 is highly expressed in human medulloblastoma and that it proved to be a prognostic marker both in a training set of 43 tumors that were analyzed by immunohistochemistry as well as in a test set of 130 tumors that were independently analyzed by expression microarrays. Functionally, our experiments show that FoxM1 is required for the growth of medulloblastoma cells and that it may be targetable by the thiazole antibiotic siomycin A.

The clinical outcome of patients suffering from medulloblastoma is very heterogeneous with a 5-year event-free survival rate ranging from only 20% to 40% for infants with metastatic medulloblastoma to more than 80% for patients with nonmetastatic medulloblastoma (34). Nevertheless, all of these tumors are considered WHO grade IV tumors and treated with radiation and multimodal chemotherapy. This may ideally result in the survival of patients with aggressive medulloblastoma, but it also causes severe long-term side effects in "overtreated" patients with less aggressive tumors. It is therefore one of the big challenges for pathologists to reliably identify more and less aggressive medulloblastomas and to facilitate the clinician’s decision for an appropriate treatment that is based on a tumor’s individual fingerprint. Keeping this idea in mind, recent advances have finally made it possible to identify aggressive medulloblastoma that are associated with a poor clinical outcome (3). However, current approaches to characterize medulloblastoma are mainly based on global gene expression arrays, and single prognostic markers that may be easily evaluated on formalin-fixed, paraffin-embedded material are rare. Nuclear expression of β-catenin is one example for such a marker. It has, in recent years, been identified and confirmed to correlate with relatively good outcome (35) and may therefore be implemented in future risk stratifications prior to treatment decisions (36). Expression of FoxM1 is easily detectable by antibody staining on paraffin sections and has been proven as a predictive marker both in a smaller training set of medulloblastoma samples and in a larger test set. Our results are in line with previous reports on glioma (22), lung cancer (37), and malignant peripheral nerve sheath tumors (38) that have also suggested the
expression of FoxM1 as a marker of poor clinical outcome.

Whereas the expression of FoxM1 is dispensable for the normal proliferation of granule neurons and for the overall development of the cerebellum (11), we show here that medulloblastoma cells undergo mitotic catastrophe and stop growing in the absence of FoxM1. These observations suggest that expression of FoxM1 is essentially required in cancer but may be compensated by other factors during normal cerebellar development, a major requirement for the treatment of children with drugs that suppress the expression of FoxM1. Siomycin A has been identified to specifically downregulate the expression of FoxM1 (33) and is therefore discussed as a potential candidate to treat patients with tumors that express high levels of FoxM1 (39). Indeed, recent studies showed that thiazole antibiotics, such as siomycin A, can suppress tumor growth in a human breast cancer xenograft model and may have potential anticancer activities against breast cancer in vitro (40). It will therefore be interesting to see whether future studies may uncover similar effects in mouse models for medulloblastoma and in clinical studies.

References


Expression of FoxM1 Is Required for the Proliferation of Medulloblastoma Cells and Indicates Worse Survival of Patients

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