FoxM1B Is Overexpressed in Human Glioblastomas and Critically Regulates the Tumorigenicity of Glioma Cells

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Abstract
The transcription factor Forkhead box M1 (FoxM1) is overexpressed in malignant glioma. However, the functional importance of this factor in human glioma is not known. In the present study, we found that FoxM1B was the predominant FoxM1 isoform expressed in human glioma but not in normal brain tissue. The level of FoxM1 protein expression in human glioma tissues was directly correlated with the glioma grade. The level of FoxM1 protein expression in human glioblastoma tissues was inversely correlated with patient survival. enforced FoxM1B expression caused SW1783 and HS683 glioma cells, which do not form tumor xenografts, to regain tumorigenicity in nude mouse model systems. Moreover, gliomas that arose from FoxM1B-transfected anaplastic astrocytoma SW1783 cells displayed glioblastoma multiforme phenotypes. inhibition of FoxM1 expression in glioblastoma U-87MG cells suppressed their anchorage-independent growth in vitro and tumorigenicity in vivo. Furthermore, we found that FoxM1 regulates the expression of Skp2 protein, which is known to promote degradation of the cell cycle regulator p27Kip1. These results showed that FoxM1 is overexpressed in human glioblastomas and contributes to glioma tumorigenicity. Therefore, FoxM1 might be a new potential target of therapy for human malignant gliomas. (Cancer Res 2006; 66(7): 3593-602)

Introduction
Gliomas encompass all primary central nervous system tumors of glial cell origin. The most common form of glioma in humans is astrocytoma, which, according to the WHO classification (1), comprises pilocytic astrocytoma (grade 1), low-grade astrocytoma (grade 2), anaplastic astrocytoma (grade 3), and glioblastoma multiforme (grade 4). Pilocytic astrocytoma is the least malignant type of astrocytoma, occurs mainly in children, and only very rarely progresses to a more malignant tumor type. In contrast, patients with low-grade astrocytoma or anaplastic astrocytoma frequently experience malignant progression to glioblastoma multiforme. Despite recent advances in both diagnostic modalities and therapeutic strategies, glioma remains one of the deadliest human cancers. The 5-year survival rate in patients with glioma is among the lowest for all cancers (2, 3). In patients with glioblastoma multiforme, the median survival duration ranges from 9 to 12 months (3). Advances in the treatment of malignant glioma will require an improvement in the understanding of the biology and molecular mechanisms of glioma development and progression. Recently, several studies have characterized gene expression profiles associated with glioblastoma using oligonucleotide-based microarray analysis and real-time reverse transcription-PCR (RT-PCR). In particular, they have identified a set of candidate genes whose differential expression probably plays a role in glioma progression. Remarkably, two of these studies found that the level of expression of the gene encoding Forkhead box M1 (FoxM1) was significantly higher in glioblastomas than in low-grade astrocytoma (4, 5).

FoxM1 (previously known as HFH-11, WIN, MPP2, and Trident) is a member of the Fox transcription factor family (6–10). FoxM1 is predominantly expressed at the mRNA level in fetal tissue, whereas its expression is extinguished in differentiated cells (6, 9). Several lines of evidence suggest that FoxM1 is a key cell cycle regulator of both the transition from G1 to S phase and progression to mitosis (11–16). For example, several studies showed that FoxM1 is essential for mediating G2–M progression and chromosome segregation (12–14) and that loss of FoxM1 causes centrosome amplification and mitotic catastrophe (15). Furthermore, FoxM1 has been shown to regulate transcription of cell cycle genes essential for G1–S and G2–M progression and chromosome stability and segregation, including Nek2, KIF20A, CENP-A, CENP-F, CDc25A, CDc25B, p27Kip1, cyclin B, and cyclin D1 (11–19).

FoxM1 may contribute to oncogenesis in some tissues. For example, FoxM1 expression was found to be up-regulated in basal cell carcinomas, hepatocellular carcinoma, and primary breast cancer (15, 18, 19). Also, mice with FoxM1 conditionally deleted from hepatocytes were found to be highly resistant to hepatocellular carcinoma development in response to liver tumor induction by diethylnitrosamine and phenobarbital (19). Moreover, FoxM1 was identified as a novel target of human papillomavirus type 16 E7 protein, which may be important for transformation (20). These results suggested that FoxM1 plays a role in the oncogenesis of some malignancies. However, whether FoxM1 expression contributes to glioma development and progression is not known. In the present study, we sought to determine whether and, if so, how FoxM1 regulates the growth of glioma. We found for the first time that aberrant FoxM1 expression directly affects the tumorigenicity of human gliomas.

Materials and Methods
Primary human glioma specimens and immunohistochemical analyses. Human glioma specimens were obtained from The University of Texas M.D. Anderson Cancer Center after approval of this study by the...
institutional review board. The database of University of Texas M.D. Anderson Cancer Center clinicopathologic characteristics was used. All tumors were from patients with a newly diagnosed glioma who had received no therapy before sample collection. All glioblastoma multiforme patients underwent gross total resection of their tumors and had completed conventional external beam radiation therapy after surgery. Overall survival time of the glioblastoma multiforme patients was measured from the date of diagnosis to the date of death. Sections of paraffin-embedded glioma specimens were stained with a polyclonal antibody against human FoxM1 (MPPP 2 K-19; Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:100 using standard procedures (18, 20). Similar tissue sections immunostained with nonspecific IgG were used as negative controls. Staining was classified using a three-tiered system according to the percentage of positive cells and staining intensity as described previously (21): negative (0/+), moderate positive (+/+), or strong positive (++/+). Scoring of staining intensity was conducted in a blinded manner to prevent bias from knowledge of clinical data.

**Semiquantitative RT-PCR.** Normal brain and glioma tissue specimens were stored frozen at −75°C until use. RNA was isolated from frozen tissue specimens with TRizol (Invitrogen, San Diego, CA). After oligo(dT)-primed reverse transcriptase of 500 ng total RNA was done, the resulting single-stranded cDNA was amplified using Taq DNA polymerase (Promega, Madison, WI). The primers used were 5′-GGGGCCAGGCAGGGAGATGAA-3′ (forward primer) and 5′-CCACTCTCTCAAGGGAGGGCTC-3′ (reverse primer) for human FoxM1 and 5′-TGGGAGAATGGTGAAGTTGCG-3′ (forward primer) and 5′-CTGGAAGGTGTGATGGGGA-3′ (reverse primer) for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Aliquots (10 μL) of the amplification products were separated by electrophoresis through a 1.5% agarose gel and visualized by ethidium bromide staining. Quantitative analysis was done with the use of densitometry and standardized with reference to the GAPDH reading.

**Cell lines and culture conditions.** The human glioma cell line HS683, anaplastic astrocytoma cell lines SW1088 and SW1783, and glioblastoma cell lines U-118 MG, LN-229, and U-87 MG were obtained from the American Type Culture Collection (Manassas, VA). The glioblastoma cell line HF U-251 MG (22) and immortalized normal human astrocyte line American Type Culture Collection (Topsham, ME) to calculate the percentage of cells at G1 and S phase.

**Animals.** Female athymic BalB/c nude mice were purchased from the Animal Production Area of the National Cancer Institute, Frederick Cancer Research Facility (Frederick, MD). The mice were housed in laminar flow cabinets under specific pathogen-free conditions and used at 8 weeks of age. The animals were maintained according to institutional regulations in facilities approved by the Association for Assessment and Accreditation of Laboratory Animal Care in accordance with current regulations and standards of the U.S. Department of Agriculture, Department of Health and Human Services, and NIH.

**Northern blot analysis.** Cellular mRNA was extracted from glioma cells using the FastTrack mRNA isolation kit (Invitrogen), fractionated on a 1% denaturing formaldehyde agarose gel, electrotransferred to a nylon membrane, and crosslinked with UV light. Northern hybridization was done by using [32P]dCTP-labeled FoxM1 cDNA probes. Equal mRNA loading was monitored by hybridizing the same membrane with a β-actin cDNA probe.

**Western blot analysis.** Whole cell lysates or nuclear proteins were prepared from glioma cells (24). Standard Western blotting was done with a polyclonal rabbit antibody against human FoxM1 (MPPP 2 K-19; Santa Cruz Biotechnology) or anti-p27, anti-Skp2, and anti-cyclin D1 antibodies (Sigma Chemical Co., St. Louis, MO) and used as loading controls. The probe proteins were detected using the Amersham enhanced chemiluminescence system according to the instructions of the manufacturer.

**Stable transfection of glioma cells.** To generate the pcDNA3.1-FoxM1B plasmid, full-length human FoxM1B was released by EcoRI and XhoII digestion of the cytomegalovirus human FoxM1B cDNA expression vector (6) and subcloned into the pcDNA3.1 (Invitrogen). To generate a FoxM1- 

**Analysis of cell cycle distribution.** Ethanol-fixed cells were treated with RNase and stained with propidium iodide (100 μg/mL in PBS). The stained cells were detected using flow cytometry (Becton Dickinson Immunocytometry Systems, San Jose, CA). Data were analyzed with a cell cycle analysis software program (ModFit LT version 2.8; Verity Software House, Topsham, ME) to calculate the percentage of cells at G1 and S phase.

**Colony formation in soft agar.** SW1783 and HS683 cells were transfected with 3 μg pcDNA3.1-FoxM1B or control vector pcDNA3.1. U-87MG cells were transfected with the FoxM1-siRNA oligomucleotide (100 nmol/L) or control siRNA (100 nmol/L). Twenty-four hours after transfection, cells were trypsinized, and 5 × 103 cells were mixed with 0.3% agar solution in DMEM containing 10% FBS and 200 μg/mL neomycin and layered on top of a 0.66% agar layer in six-well tissue culture plates. Plates were incubated at 37°C in a humidified atmosphere containing 5% CO2 for 14 days. Colonies were then stained with p-iodonitrotetrazolium violet (1 mg/mL) and examined microscopically. Only colonies containing >50 cells were scored. These were reported as the mean number of colonies observed in 10 randomly chosen microscope fields.

**S.c. tumor growth.** To study the kinetics of tumor formation, glioma cells (3 × 105 or 1 × 106 cells in 0.1 mL of HBSS) were injected s.c. into nude mice (24). The diameter of the resulting tumors was measured once every 5 days. The latency of tumor formation was determined according to the number of days from glioma cell injection to the observation of a tumor having a maximum diameter >3 mm.

**Intracranial human glioma xenograft model.** Glioma cells (1 × 106) were injected intracranially into nude mice as described previously (25). Two independent experiments with five mice per group in each experiment were done. Animals showing general or local symptoms were killed; the remaining animals were killed 90 days after glioma cell injection. The brain of each mouse was harvested, fixed in 4% formaldehyde, and embedded in paraffin. Tumor formation and the phenotype were determined by histologic analysis of HE- and stained sections.

**Histology and immunohistochemistry of xenograft tumors.** Brain tissue specimens were fixed by immersion in neutral buffered formalin, embedded in paraffin, sectioned, and stained with H&E according to standard protocols. Tissue sections were immunostained using anti-mouse CD34 (Novoceastra Laboratories, Norwell, MA) to detect the CD34 antigen in endothelial cells.

**Statistics.** The significance of the data from patient specimens was determined using the χ2 test. The significance of the in vitro results was determined using Student's t test (two-tailed), whereas the significance of the in vivo data was determined by using the Mann-Whitney U test. Kaplan-Meier survival analysis was used to compare overall survival times of glioblastoma multiforme patients.

**Results**

FoxM1B is the main FoxM1 isoform in human gliomas. The structure of the FoxM1 gene includes nine exons, two of which (A1 and A2) are alternatively expressed, giving rise to at least two
differentially spliced isoforms (FoxM1A and FoxM1B; ref. 6). To
determine the relative expression of these two isoforms in human
normal brain and glioma tissues (consisting of specimens of low-
grade astrocytoma, anaplastic astrocytoma, and glioblastoma
multiforme), we used semiquantitative RT-PCR analysis with
specific primers spanning exons A1 and A2. As shown in Fig. 1A,
we found predominant expression of FoxM1B (358 bp) transcripts
in the glioma specimens. Sequencing analysis confirmed that the
major band in specimens 13 and 14 was FoxM1B; the rest of the
bands were nonspecific products (data not shown). We did not
detect expression of FoxM1A (472 bp) transcripts in any of the
glioma samples. Moreover, the levels of FoxM1 RNA in the
glioblastoma multiforme specimens were twice as high as those
in the anaplastic astrocytoma specimens ($P < 0.01$) and 3.5 to 8.5
times higher than those in the low-grade astrocytoma specimens
($P < 0.001$). We did not detect FoxM1 RNA expression in normal
brain tissue specimens.

Expression of FoxM1 directly correlates with the grade of
glioma. We studied the expression level and intracellular
localization of the FoxM1 protein in three normal brains, 25
low-grade astrocytomas, 34 anaplastic astrocytomas, and 50 glioblastoma
multiforme specimens. We did immunohistochemical
staining on these samples using an antibody specific for FoxM1
(20). We found FoxM1 immunoreactivity in both the cytoplasmic
and nuclear compartments (Fig. 1B). Figure 1B shows tissue
sections displaying strong staining (+++), moderate staining (++),
and negative staining (0/+). Figure 1C shows that 4% of the
low-grade astrocytomas (only 1 of 25 tumors) were strongly
positive, 4% were moderately positive, and 92% were negative for
FoxM1 expression. Also, 14.7% of the anaplastic astrocytomas were

Figure 1. Expression of FoxM1 RNA in
human normal brain and glioma tissues. 
A, FoxM1A and FoxM1B mRNA
expression by semiquantitative RT-PCR
analysis. Fold was calculated relative to
sample 1 (lane 3). MW, molecular weight;
NB, normal brain tissue; LGA, low-
grade astrocytoma; AA, anaplastic astrocytoma;
GBM, glioblastoma multiforme. B, FoxM1
protein expression. Immunohistochemical
staining was done with an anti-FoxM1
antibody on archival tissue sections. 0/+, 
representative sections of weak or no
staining; ++, moderate staining; +++,
strong staining (magnification, ×200).
Solid arrowheads, positive cytoplasmic
staining; open arrowheads, nuclear
staining (inset magnification, ×400). 
C, percentage of tumors strongly positive,
moderately positive, and negative for
FoxM1 in each group. Significantly higher
levels of FoxM1 expression were observed
in glioblastoma multiformes than in
anaplastic astrocytomas ($^{*}P < 0.01$) and
low-grade astrocytomas ($^{*}P < 0.01$)
according the $\chi^2$ test. D, graph showing
Kaplan-Meier estimates of overall survival
time in patients who had a glioblastoma
multiforme with negative, moderate, or
strong FoxM1 expression. The survival
time of patients who had a glioblastoma
multiforme with negative FoxM1
expression was significantly longer than
that of patients who had a glioblastoma
multiforme showing moderate or strong
FoxM1 expression ($P < 0.001$).
strongly positive, 26.5% were moderately positive, and 58.8% were negative for FoxM1 expression. In contrast, 36% of the glioblastoma multiformes were strongly positive, 36% were moderately positive, and 28% were negative for FoxM1 expression. Analysis of the positive staining data showed significantly higher levels of FoxM1 expression in glioblastoma multiformes than in anaplastic astrocytomas \((P < 0.01)\) and low-grade astrocytomas \((P < 0.01)\). We did not detect FoxM1 protein expression in normal brain tissues. Thus, the human glioma tissue specimens apparently had a substantially higher level of FoxM1 expression than normal tissue and this expression correlated directly with the grade of the glioma.

Increased expression of FoxM1 in glioblastoma multiforme was associated with decreased patient survival. We next investigated the relationship of FoxM1 expression and patient survival in the 50 glioblastoma multiformes. The median survival durations in patients with strongly positive, moderately positive, and negative staining of FoxM1 were 32, 57, and 113 weeks, respectively. Kaplan-Meier survival curves indicated that increased expression of FoxM1 was significantly associated with poor overall survival of glioblastoma multiforme patients \((P < 0.001; \text{Fig. 1D})\).

Glioma cell lines express FoxM1 at various levels. We next analyzed the expression of FoxM1 mRNA and protein in the immortalized normal human astrocyte cell line NHA-E6/E7/hTERT and glioma cell lines Hs683 (26, 27), anaplastic astrocytoma cell lines SW1088 and SW1783, and glioblastoma cell lines U-118 MG, LN-229, U-87 MG, and HF U-251 MG. We used immortalized NHAs because the existence of low-grade astrocytoma cell lines has not yet been reported. Significantly higher expression of FoxM1 mRNA and protein was evident in U-118 MG, LN-229, U-87 MG, and HF U-251 MG glioblastoma cells than in SW1088, SW1783, and Hs683 cells. Immortalized normal human astrocytes had a negligible level of FoxM1 mRNA and protein expression (Fig. 2A).

**Effect of altered FoxM1 expression on anchorage-independent growth of human glioma cells.** We determined whether overexpression of FoxM1B in human glioma cells affects anchorage-independent growth, a hallmark of the transformation phenotype. We transiently transfected SW1783 and Hs683 cells, which have low levels of FoxM1 expression, with the FoxM1B expression vector pcDNA3.1-FoxM1B or control vector pcDNA3.1-Neo. As shown in Fig. 2B, transfection of FoxM1B in SW1783 and Hs683 cells caused a significant increase in anchorage-independent growth as evidenced by an increase in the number of colonies on soft agar compared with control vector–transfected cells. These results suggested that enforced FoxM1B expression increases the anchorage-independent growth of human glioma cells.

Conversely, we determined whether knocking down FoxM1 expression affects anchorage-independent growth using FoxM1-siRNA. We transiently transfected U-87MG cells, which have higher levels of FoxM1 expression, with 100 nmol/L FoxM1-siRNA oligonucleotide or control siRNA. The resultant suppression of FoxM1 expression substantially inhibited the anchorage-independent growth of these human glioma cells (Fig. 2B).

**FoxM1B overexpression increases the tumorigenicity of human glioma cells.** To determine whether FoxM1 plays an important role in the tumorigenicity of human glioma cells, we transfected FoxM1B into SW1783 and Hs683 cells, neither of which is tumorigenic in nude mice (26–28). To avoid clonal selection and variation, we carried out three independent transfections of pcDNA3.1-FoxM1B in both cell lines and pooled G418-resistant colonies to establish stable transfectants, designated as...
FoxM1B-transfected SW1783 cell lines (SW1783-FoxM-a, SW1783-FoxM-b, and SW1783-FoxM-c) and Hs683 cell lines (Hs683-FoxM-a, Hs683-FoxM-b, and Hs683-FoxM-c). Thus, each of the stably transfected cell lines represented a pooled culture of cells. Western blot analysis showed increased levels of FoxM1 protein expression in FoxM1B-transfected SW1783 and Hs683 cells relative to their untransfected counterparts (Fig. 3A).

We first studied the kinetics of glioma growth using a s.c. xenograft nude mouse model system. As shown in Fig. 3B, parental and control vector–transfected SW1783 cells (3 × 10⁶ per mouse) formed tiny masses (<3.6 mm in diameter) in nude mice by day 5 after injection. However, the masses shrank afterward and disappeared within 15 days. The masses did not grow back over the following 8 weeks (data not shown); thus, the mass formation could have been the result of an inflammatory reaction to the tumor cell injections. In contrast, FoxM1B-transfected SW1783 cells (SW1783-FoxM-a, SW1783-FoxM-b, and SW1783-FoxM-c; 3 × 10⁶ per mouse) formed aggressively growing tumors that reached a diameter of >10 mm within 40 days after tumor cell injection. We found similar results using parental, control vector–transfected, and FoxM1B-transfected Hs683 cells (1 × 10⁶ per mouse; Fig. 3B). These results showed that FoxM1 overexpression increases the tumorigenicity of human glioma cells.

Next, to study the growth of these glioma cells in the brain, we used an orthotopic xenograft model of human glioma in which we injected glioma cells intracranially into nude mice. As with the s.c. injected cells, intracranially implanted SW1783 and SW1783-Neo cells (1 × 10⁶ per mouse) did not form tumors at 90 days after injection (Table 1). In contrast, intracranially injected FoxM1B-transfected SW1783 cells (1 × 10⁶ per mouse) did form brain tumors. Intracranial injection of SW1783-FoxM1B cells into nude mice also significantly shortened their survival time (range, 26-34 days; P < 0.001). We observed similar results with the use of FoxM1B-transfected Hs683 cells (Table 1). These results showed that FoxM1B overexpression directly promotes the growth of human glioma cells in the brain in nude mice.

We then examined the histologic features of gliomas that arose from SW1783-FoxM1B cells in the brain in nude mice. Microscopically, these lesions were highly cellular, displayed nuclear pleomorphism, were prominently mitotic (Fig. 3C1), and were highly invasive (Fig. 3C2). They also had microvascular proliferation characterized by a substantially increased number of CD34-positive microvessels (Fig. 3C3). Furthermore, the lesions contained pseudopalisading astrocytoma cells around areas of necrosis (Fig. 3C4), one of the most characteristic morphologic features of human glioblastoma multiforme (29, 30). These features are compatible with the pathologic diagnosis of glioblastoma multiforme according to the WHO classification scheme for human astrocytoma. These results suggested that overexpression of FoxM1B in SW1783 anaplastic astrocytoma cells renders the formation of glioblastoma-like tumors.

**Inhibition of FoxM1 expression suppresses glioma growth in the brain in nude mice.** To further determine the effect of altered FoxM1 expression on glioma growth, we established two stable FoxM1 siRNA–transfected U-87MG cell lines (U-87MG-FoxM1-siRNA-a and U-87MG-FoxM1-siRNA-b) by performing duplicate transfection experiments with the U-87MG cell line. After undergoing G418 selection for 14 days, less colonies survived in cells transfected with FoxM1 siRNA than with control siRNA. Colonies surviving within each respective transfection group were pooled to establish the stably transfected cell lines. Western blot analysis showed decreased levels of FoxM1 protein expression in the FoxM1 siRNA–transfected U-87MG cell lines (Fig. 4A). We then intracranially injected these cells into nude mice (1 × 10⁶ per mouse) to evaluate the effect of FoxM1 knock down on brain tumor growth. U-87MG and U-87MG-control-siRNA cells produced brain tumors in all of the injected mice (Table 1). The mice became moribund around 31 days after the injection. In contrast, except for a small tumor in one of the mice, the FoxM1 siRNA–transfected U-87MG cells produced no brain tumors, which resulted in increased overall survival time (P < 0.001). These results showed that inhibition of FoxM1 expression by FoxM1-siRNA significantly suppresses the tumorigenicity of human glioblastoma cells.

**Effect of altered FoxM1 expression on glioma cell cycle progression in vitro.** Defects in the regulation of cell cycle progression are thought to be among the most common features of glioblastoma multiforme (31). Previous studies have suggested that FoxM1 is required for proper cell cycle function (11–16). Therefore, we used flow cytometry to assess whether increased tumorigenicity of FoxM1B-transfected glioma cells was associated with alterations in cell cycle progression. As shown in Fig. 4B, the percentage of FoxM1B-transfected SW1783 cells in S phase was increased compared with that of parental and control vector–transfected SW1783 cells. We found similar results with FoxM1B-transfected, parental, and control vector–transfected Hs683 cells (Fig. 4B). Conversely, the percentage of FoxM1 siRNA–transfected U-87MG cells in S phase was decreased relative to that of parental and control siRNA–transfected U-87MG cells (Fig. 4B). These results indicated that FoxM1B overexpression accelerates cell cycle progression of glioma cells, whereas down-regulation of FoxM1 expression inhibits it.

**Effect of altered FoxM1 expression on the protein level of Skp2, p27Kip1, and cyclin D1.** To identify the mechanisms of acceleration of glioma cell cycle progression by FoxM1B overexpression, we examined the expression of several cell cycle regulatory genes. Because p27Kip1 protein is a major regulator of the G1 to S phase transition and is active in the nucleus, we used Western blot analysis to examine whether FoxM1B influences expression of p27Kip1 in the nucleus. As shown in Fig. 5A, overexpression of FoxM1B in SW1783 and Hs683 cells produced a 3- to 4-fold decrease in the level of nuclear p27Kip1 compared with that in parental and control vector–transfected SW1783 and Hs683 cells. However, overexpression of FoxM1B in SW1783 and Hs683 cells did not affect the mRNA level of p27Kip1 (data not shown). In contrast, the level of expression of Skp2 protein, the specific recognition factor for p27Kip1 ubiquitination, was significantly increased in FoxM1B-transfected SW1783 and Hs683 cells (Fig. 5B). In comparison, the level of nuclear p27Kip1 expression in FoxM1B siRNA–transfected U-87 MG cells was significantly increased compared with that in parental and control vector–transfected SW1783 and Hs683 cells (Fig. 5D). In contrast, the level of Skp2 protein expression in FoxM1B siRNA–transfected U-87 MG cells was significantly decreased compared with that in parental and control siRNA–transfected cells (Fig. 5C), whereas the level of Skp2 protein expression in FoxM1B siRNA–transfected U-87 MG cells was significantly decreased compared with that in parental and control vector–transfected U-87 MG cells (Fig. 5C). Furthermore, we observed that increased expression of FoxM1 in SW1783 cells achieved by transient transfection of various concentrations of FoxM1 expression vector led to increased expression of Skp2 in a dose-dependent manner and that increased Skp2 expression seemed to correlate with decreased levels of p27Kip1 protein (Fig. 5D). Increased expression of FoxM1 in these cells also correlated with increased Skp2 and decreased p27Kip1 protein expression in a time-dependent manner (Fig. 5D). These effects were not observed in control vector–transfected cells (Fig. 5D).
Additionally, the protein level of cyclin D1 was significantly increased in the FoxM1B-transfected SW1783 and Hs683 cells (Fig. 5B) but was significantly decreased in FoxM1 siRNA–transfected U-87MG cells (Fig. 5C). Therefore, these data suggested that FoxM1 expression regulates the expression of these multiple cell cycle genes in glioma cells.

**Discussion**

Although previous studies have suggested that FoxM1 plays a role in basal cell carcinoma, carcinogenesis of hepatocellular carcinoma, and breast cancer (15, 18, 19), its role and molecular mechanisms in glioma are not known. In the present study, we found both clinical and causal experimental evidence that aberrant FoxM1 expression critically regulates the tumorigenicity of human glioma cells. We also provided evidence that FoxM1 regulates the expression of Skp2 protein, suggesting a potential mechanism for acceleration of the cell cycle by altered FoxM1 expression in human glioma.

Previously, in a microarray study, FoxM1 mRNA was found to occur at a higher level in anaplastic astrocytoma and glioblastoma multiforme specimens than in astrocytoma grade 2 specimens (4).
High levels of FoxM1 mRNA were also noted in glioblastoma multiforme in a study comparing the expression profiles of glioblastoma multiformes with those of grade 1 and grade 2 astrocytomas and normal brain tissue (5). Also, a number of FoxM1 isoforms have been identified (6). In the present study, we found that FoxM1B mRNA is the predominant FoxM1 isoform in human gliomas. We also examined the expression of FoxM1 in glioma cells per se, as different types of gliomas may contain different numbers of infiltrating normal cells. Immunohistochemical analyses indicated that the FoxM1 protein expression level is higher in glioblastoma multiformes than in anaplastic astrocytomas and low-grade astrocytomas.

Hyperproliferation is a hallmark of glioblastoma multiforme (31). In the present study, FoxM1B overexpression increased the growth of glioma cells both in vitro and in vivo, which was at least partially caused by accelerated glioma cell cycle progression. By using RNA interference with a transient transfection method, Leung et al. (16) showed that reducing FoxM1 expression caused an increase in the population of breast cancer cell lines at G2 phase 48 hours after transfection. In the present study, using established, stable siRNA-transfected glioma cells, we found that reduced FoxM1 expression caused a decrease in the population of U-87MG cells at S phase 16 hours after plating the cells in fresh medium with serum. The discrepancies in these results may relate to differences in the methods and/or time points used. Indeed, when we transiently transfected U-87MG cells with a FoxM1-siRNA oligonucleotide and analyzed their cell cycle progression after 48 hours, we found an increased cell population at G2 phase compared with control siRNA-transfected cells (data not shown). Nevertheless, these data are consistent with the notion that FoxM1 protein has been established as a key regulator of both the G1-S phase transition and G2-M progression (11–16).

Table 1. Effect of FoxM1 overexpression and knockdown on glioma growth in the brain of nude mice

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Incidence of tumor formation</th>
<th>Survival median days (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SW1783</td>
<td>0/5</td>
<td>90 (All &gt;90)</td>
</tr>
<tr>
<td>SW1783-Neo</td>
<td>0/5</td>
<td>90 (All &gt;90)</td>
</tr>
<tr>
<td>SW1783-FoxM-a</td>
<td>5/5</td>
<td>26 (21-30)*</td>
</tr>
<tr>
<td>SW1783-FoxM-b</td>
<td>5/5</td>
<td>32 (26-34)*</td>
</tr>
<tr>
<td>SW1783-FoxM-c</td>
<td>4/4</td>
<td>28 (27-30)*</td>
</tr>
<tr>
<td>Hs683</td>
<td>0/5</td>
<td>90 (All &gt;90)</td>
</tr>
<tr>
<td>Hs683-Neo</td>
<td>0/5</td>
<td>90 (All &gt;90)</td>
</tr>
<tr>
<td>Hs683-FoxM-a</td>
<td>5/5</td>
<td>27 (26-29)*</td>
</tr>
<tr>
<td>Hs683-FoxM-b</td>
<td>5/5</td>
<td>35 (29-40)*</td>
</tr>
<tr>
<td>Hs683-FoxM-c</td>
<td>5/5</td>
<td>30 (27-35)*</td>
</tr>
<tr>
<td>U-87MG</td>
<td>5/5</td>
<td>26 (23-31)</td>
</tr>
<tr>
<td>U-87MG-Control-siRNA</td>
<td>5/5</td>
<td>22 (21-25)</td>
</tr>
<tr>
<td>U-87MG-FoxM1-siRNA-a</td>
<td>0/5</td>
<td>90 (All &gt;90)*</td>
</tr>
<tr>
<td>U-87MG-FoxM1-siRNA-b</td>
<td>1/5</td>
<td>86 (72 to &gt;90)*</td>
</tr>
</tbody>
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NOTE: Glioma cells (1 × 10⁶ cells) were implanted intracranially into nude mice. Mice were killed when they were moribund or on day 90. Brains were harvested and tumor formation was determined by histology. *P < 0.001. Results were shown for one representative experiment of two.

In addition, we found that FoxM1 overexpression diminished the expression of nuclear p27Kip1 protein but increased the expression of Skp2 and cyclin D1 protein. Previous studies have shown that nuclear expression of p27Kip1 decreases with malignancy in human astrocytic gliomas and that p27Kip1 has independent prognostic value in patients with malignant glioma (32–34). The p27Kip1 gene is a major regulator of the cell cycle and a potent tumor suppressor gene (35). p27Kip1 exerts its suppressive effect through cyclin-dependent kinase-cyclin complexes by inhibiting the phosphorylation of pRb by these complexes, which in turn arrests cells at G1.
phase and prevents them from entering S phase. Deregulated expression of p27Kip1 plays a critical role in the pathogenesis of many human tumors (36). However, mutations of the p27Kip1 gene seem to be extremely rare in human malignancies (36). Decreased expression of p27 protein seems to be caused by increased ubiquitin-mediated degradation. Recently, Skp2 was shown to mediate p27Kip1 degradation as a specific substrate-recognition subunit and to have oncogenic properties (37). In human gliomas, the Skp2 expression level is directly correlated with the tumor grade but inversely correlated with the p27Kip1 level (38). In the present study, we found that FoxM1B overexpression in glioma cells significantly decreased the p27Kip1 protein level in the nucleus but did not alter the p27Kip1 mRNA level, which is consistent with previous studies (19). Also, we found that FoxM1B overexpression significantly increased Skp2 expression. Therefore, FoxM1 probably regulates p27Kip1 protein expression indirectly by inducing Skp2 expression, which mediates the degradation of p27Kip1 protein. Finally, because p27Kip1 has been implicated in modulation of apoptosis of various types of cells, including glioblastoma multiforme cells (39, 40), altered expression and function of FoxM1B might affect apoptosis, the mechanism of which warrants further investigation.

Several studies have reported that cyclin D1 expression is correlated significantly with the degree of malignancy in astrocytomas and that glioblastoma multiformes have increased cyclin D1 expression (41, 42). Interestingly, the human cyclin D1 gene was originally cloned from the glioblastoma multiforme cell line U-118 MG (42). In the present study, we found that FoxM1B overexpression in glioma cells significantly increased cyclin D1 expression, which was consistent with findings with other types of cells (11, 13). Thus, the molecular mechanisms by which FoxM1 regulates the growth of glioma cells are associated with alterations in p27Kip1, Skp2, and cyclin D1 expression.

Our results indicate that FoxM1 is an attractive target for glioblastoma therapy. FoxM1 siRNA–transfected U-87MG cells with reduced expression of FoxM1 produced no brain tumors.
in most of the mice injected with them, although we did not achieve a complete loss of FoxM1 expression with stable FoxM1-siRNA transfection. Moreover, gliomas that arose from FoxM1B-transfected anaplastic astrocytoma SW1783 cells displayed characteristics of the glioblastoma multiforme phenotype, such as necrosis with pseudopalisading. Focal necrosis is a key histopathologic feature of glioblastoma multiforme (29, 30). Glioblastoma multiforme is differentially diagnosed from low-grade astrocytomas based on the histologic presence of focal necrosis and associated microvascular proliferation. In fact, we observed that FoxM1 expression in glioblastoma multiforme is higher than that in anaplastic astrocytoma. Thus, these results imply that FoxM1 may play a role in glioma progression, although more studies are needed to clearly address this issue.

The mechanisms responsible for overexpression of FoxM1 in malignant glioma are not known. Investigators have shown that FoxM1 expression can be induced by diverse stimuli, such as liver regeneration, keratinocyte growth factor, and oxidative stress (6). FoxM1 expression can be induced by diverse stimuli, such as liver regeneration, keratinocyte growth factor, and oxidative stress (6).


# FoxM1B Is Overexpressed in Human Glioblastomas and Critically Regulates the Tumorigenicity of Glioma Cells


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