MELK-Dependent FOXM1 Phosphorylation is Essential for Proliferation of Glioma Stem Cells

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ABSTRACT

Glioblastoma multiforme (GBM) is a life-threatening brain tumor. Accumulating evidence suggests that eradication of glioma stem-like cells (GSCs) in GBM is essential to achieve cure. The transcription factor FOXM1 has recently gained attention as a master regulator of mitotic progression of cancer cells in various organs. Here, we demonstrate that FOXM1 forms a protein complex with the mitotic kinase MELK in GSCs, leading to phosphorylation and activation of FOXM1 in a MELK kinase-dependent manner. This MELK-dependent activation of FOXM1 results in a subsequent increase in mitotic regulatory genes in GSCs. MELK-driven FOXM1 activation is regulated by the binding and subsequent trans-phosphorylation of FOXM1 by another kinase PLK1. Using mouse neural progenitor cells (NPCs), we found that transgenic expression of FOXM1 enhances, while siRNA-mediated gene silencing diminishes neurosphere formation, suggesting that FOXM1 is required for NPC growth. During tumorigenesis, FOXM1 expression sequentially increases as cells progress from NPCs, to pretumorigenic progenitors and GSCs. The antibiotic Siomycin A disrupts MELK-mediated FOXM1 signaling with a greater sensitivity in GSC compared to neural stem cell. Treatment with the first-line chemotherapy agent for GBM, Temozolomide, paradoxically enriches stem cell properties of GSCs, suggesting that FOXM1 is required for NPC growth. Therefore, there is an urgent need to develop novel therapies to effectively target resistant GBM cells.

INTRODUCTION

Glioblastoma multiforme (GBM) is the most common primary malignant brain tumor and is highly aggressive and therapy-resistant [1–3]. Even patients with well-demarcated tumors in noneloquent areas that allow for gross-total resection at surgery and who respond well to first-line chemotherapy and radiotherapies frequently do not escape from subsequent recurrence and ultimately die from disease progression. Therefore, there is an urgent need to develop novel therapies to effectively target resistant GBM cells. GBM is composed of mixed tumor cell populations including tumors with stem cell properties, termed glioma stem-like cells (GSCs) [4, 5] Accumulating evidence suggests that stem cell properties of GSCs contribute to therapeutic resistance in GBM. Tumor stem cells are one, if not the only, cellular target in GBM, and therapeutic development targeting this subset of tumor cells may improve patient survival [6].
The transcription factor, FOXM1 is a member of the Forkhead box (FOX) family that consists of more than 50 mammalian proteins with shared homology in the winged helix DNA binding domain. Many genes within this family play critical roles in cell cycle progression and cell fate decision. Initial studies of FOXM1 characterized its role in liver development [7]. Intriguingly, FOXM1 knockout mice fail to form hepatocellular carcinoma in a carcinogenic induction model, suggesting that FOXM1 is necessary for tumor initiation in the liver [8]. Accumulated evidence suggests that FOXM1 is a proto-oncogene with elevated expression in a number of human cancers such as liver, ovarian, breast, prostate, colon, and brain tumors including GBM. Downstream target pathways of FOXM1 include vascular endothelial growth factor (VEGF), matrix metalloproteinase-2 (MMP-2), and β-catenin [9–11], each of which promotes tumor formation and progression. FOXM1 enhances cancer stem cell self-renewal through direct binding to β-catenin inducing nuclear localization and transcriptional activity [10]. Taken together, abrogation of FOXM1 signaling may provide multidirectional approaches for controlling cancers including GBM.

Maternal embryonic leucine-zipper kinase (MELK) is a serine/threonine kinase and is abundantly expressed in GBM and various other cancers. Previously, we found that MELK is highly expressed in GSCs derived from GBM samples and its mRNA expression is inversely correlated with survival in GBM patients [12]. In addition, small inhibitory RNA (siRNA)-mediated MELK abrogation induces GSC apoptosis in vitro with less inhibitory effects on normal neural progenitor cells (NPCs) [12]. A recent study using a murine breast cancer initiation model indicated that ablation of Melk eliminates tumorigenesis in vivo; thus MELK is likely a therapeutic target not only for GBM but also for other cancers [13].

Prior studies have suggested that both FOXM1 and MELK play essential roles in cell cycle progression, cancer cell growth, and maintenance of stem cell state of GBM. Furthermore, MELK and FOXM1 are highly coexpressed and coregulated in GBM suggesting that they are functionally related. Therefore, we sought to test the hypothesis that MELK and FOXM1 directly interact to regulate proliferation of GSC [14]. In this study, we interrogated the physical interaction of FOXM1 and MELK, as well as the functional roles of FOXM1-MELK signaling in GSCs. Here, we describe novel mechanistic evidence that FOXM1 cooperates with MELK to regulate the mitotic transcriptome in GSCs.

Human Specimens and Tissue Culture

Neurospheres (NS) derived from 10 GBM samples and 1 fetal brain were used in this study, as described previously [12, 15–17]. Regarding the nine samples collected at The Ohio State University (OSU), surgery was performed by I. Nakano and E.A. Chiocca in the Department of Neurological Surgery, and surgical specimens were processed for research under approved protocols (IRB Number 2005C0075). GBM157 was established in Dr. Korinblum’s laboratory at UCLA, as described previously [18]. Established GSC cultures were cultured in defined medium containing Dulbecco’s modified Eagle’s medium (DMEM)/F12/Glutamax (Invitrogen, Carlsbad, CA, http://www.invitrogen.com) supplemented with 10% fetal calf serum (Sigma Aldrich, St. Louis, MO, http://www.sigmaaldrich.com). For more detailed methods, see Supporting Information.

Vectors

All the FOXM1 vectors (wild-type and mutants, S715A, S724A, double mutant S715/T24A, S678A, T596A, TSSA, EE and the reporter 6x FOXM1) and PLK1 (wild-type and dominant negative) were kindly provided by Dr. Zheng Fu of the Virginia Commonwealth University. All MELK vectors were described previously [20].

Phosphorylation Assay

FOXM1 cDNA was inserted in enhanced green fluorescent protein (pEGFP)-C1 (Clontech, Palo Alto, CA, http://www.clontech.com) for mammalian cell expression and pet16b (Novagen) for bacterial expression. Flag-MELK and pet16b-MELK1-340 were described previously [20]. HEK293 cells were transiently transfected with the indicated plasmids, and cell lysis was performed as described [20]. The complexes were trapped with GFP-trap beads (Chromotek, Germany, http://www.chromotek.com) and analyzed for the presence of Flag-tag fused MELK with Flag antibody (Stratagene, La Jolla, CA, http://www.stratagene.com). His-MELK1-340, His-FOXM1, and His-cyclinA-CDK2 were expressed in bacteria and purified as described previously [20, 21]. His-FOXM1 was phosphorylated in vitro by His-MELK1-340, His-MELK1-340D150A, or His-cyclinA-CDK2 for 1 hour at 30°C in a buffer containing 25 mM Tris at pH 7.5, 0.1 mM [γ-32P] ATP, 2 mM magnesium acetate, and 20 mM dithiothreitol (DTT).

Gene Expression Omnibus Profile and TCGA Data

Affymetrix Human Genome U133A Array was performed as described previously. The data have been submitted to the GEO database accession number GDS1815 [22, 23]. MELK and FOXM1 expression (Affymetrix Human Genome U133A Array) data were downloaded from the GDS1815 dataset and analyzed for grade III glioma and GBM. The Cancer Genome Atlas (TCGA) data are available through the TCGA Data Portal at http://tcga-data.nci.nih.gov [24].

Drug Treatment

Siomycin A was obtained through the Developmental Therapeutics Program NCI/NIH. Siomycin A and ON-01910 (PLK1 inhibitor—selleck chemical Inc., Houston, TX, USA, http://www.selleckchem.com) stock solution was prepared using dimethyl sulfoxide (DMSO) (Sigma Aldrich). In each experiment, DMSO alone was used in control samples at a concentration in between 0.1% and 1% and identical to the concentrations of DMSO that was used in drug-treated cells in each experiment. We confirmed that the growth of the cells we used in this study is not significantly affected by DMSO at 1% or lower [18].

Statistics

Quantitative data are presented as means ± SD, unless noted otherwise in the figure legend. The numbers of replicates are noted in the figure or legends. Comparison of mean values between multiple groups was evaluated by an ANOVA followed by Tukey’s test. When multiple comparisons were involved, Holm’s method was used to adjust the multiplicities to control the type I error rate that was less than 0.05 [25]. Spearman correlation coefficients (r) were used to assess the correlation between FOXM1 and MELK expression in the STEM CELLS
GDS1815 microarray data and TCGA dataset [26]. Log-rank tests were used to compare the survival probabilities between groups in the xenograft mouse experiment. Comparison of mean values between two groups was evaluated by $\chi^2$ test or $t$ test. Log-rank analysis is used to determine statistical significance of Kaplan Meier survival curve. For all statistical methods, a $p$-value less than .05 was considered significant. For more detailed information, see Supporting Information.

### RESULTS

**FoxM1 Expression Is Restricted to NPCs in the Mouse Brain**

First, we investigated FoxM1 expression during brain ontogeny. Similar to Melk expression, FoxM1 expression in the brain was predominantly detected during early and mid-embryonic periods with a dramatic decline between embryonic day 15 (E15) and E17 (Fig. 1A). Expression of FoxM1 in the adult brain was below detectable levels by reverse transcription polymerase chain reaction (RT-PCR). We then examined FoxM1 expression in neural progenitor cultures grown as NS derived from mouse E17 cerebral cortices. FoxM1 expression was markedly high in proliferating NS, whereas its expression levels declined dramatically in the prodifferentiation—indicated by expression of neuronal and glial markers—conditions within 6 hours, the earliest time point studied (Fig. 1B). These data suggest that, similar to Melk expression, FoxM1 is preferentially expressed in proliferating murine NPCs.

We then performed immunohistochemistry of FoxM1 with mouse embryonic and adult brains. FoxM1 protein expression was restricted to the germinal zone (GZ) of embryonic day 17 (E17) brains. The majority of FoxM1+ cells were not colocalized with neuronal marker TuJ1 and vice versa (Fig. 1C).
left panels; Supporting Information Fig. S1). In contrast, we observed extensive overlap of FoxM1 (+) cells with Sox2 (+) cells in the GZ. In brains at the postnatal day 30 (P30), FoxM1 expression was restricted to the proliferative zones lining the ventricles and was not colocalized with glial fibrillary acidic protein (GFAP), suggesting that FoxM1 is unlikely to be expressed in differentiated astrocytes or type B neural stem cells (NSCs) (Fig. 1C, right panels). In addition, there was no detectable coexpression of FoxM1 and the neuroblast marker Dcx. On the other hand, FoxM1 was expressed by proliferating cells, as indicated by colocalization with BrdU. Taken together, these data suggest that the majority, if not all, FoxM1 (+) cells are rapidly proliferating type C progenitor cells in the SVZ. When FoxM1 expression was evaluated in the other neurogenic region, the subgranular zone (SGZ) of hippocampus, very few cells, if any, in the SGZ express FoxM1 at P30 (Fig. 1D).

FoxM1 Regulates NPC In Vitro

To determine the function of FoxM1 in NPCs, we assessed the effects of transgenic expression and silencing of the active FoxM1 isoform b on NS derived from E17 cerebral cortices. These neural progenitors were transfected with either the expression vectors or double-stranded RNAs designed to be siRNA for FoxM1 (isoform b) or controls. For knockdown of FoxM1, we designed two control siRNAs that silence either nontarget sequence or the transcription repressor region of FoxM1 isoform a, that is not present in the translationally active form of FoxM1, isoform b (Fig. 1E) [27]. Overexpression of FoxM1 (isoform b) resulted in a twofold enhancement of NS formation (Fig. 1E; Supporting Information Fig. S2). Although siRNA-mediated silencing of the inactive FoxM1 (isoform a) did not alter the growth of NS compared with the nontarget control, siRNA directed against the common region of FoxM1 (isoforms a and b) resulted in a significant decrease in NS numbers (Fig. 1F, right panel; Supporting Information Fig. S2). Collectively, these data suggest that FoxM1 is essential for the growth of murine NPCs derived from embryonic brains in vitro.

FoxM1 Expression Is Markedly Elevated During Gliomagenesis

Accumulating evidence suggests that GSCs and their somatic counterparts, NPCs, share various fundamental signaling pathways to regulate their self-renewal and proliferation. We therefore sought to determine the role of FoxM1 in GBM-like tumors and GSCs in mice harboring conditional inactivation of the human GBM-relevant tumor suppressors p53, Nf1, and Pten [28]. In this model, Nestin promoter-driven Bromodeoxy-yuridine (BrdU) (+) cells along the ventricular wall are the cells of origin for the cortical GBM-like tumors formed in adult mice [28]. When we compared FoxM1 staining of the SVZ of wild-type mice and genetically engineered mutant GBM mouse brains, we observed sequential elevation of FoxM1 expression along the ventricular wall during the course of tumorigenesis (Fig. 2A; Supporting Information Fig. S3). When FoxM1 expression was compared between normal brain regions versus tumor areas of individual mice, tumors had more than 200-fold higher expression than normal brain regions (Fig. 2B). We then compared FoxM1 expression in NS derived from the SVZ of wild-type mice and GBM-like tumors. FoxM1 mRNA levels were 2.5-fold greater in GBM NS than in normal NS, indicating that FoxM1 expression is elevated in GSCs compared to NPCs in vitro (Fig. 2C) [29]. The FOX family transcription factors play a crucial role in organ development and cancer initiation and propagation in a context-dependent manner [30, 31]. To determine which members of the FOX gene family are upregulated in GSCs, we evaluated expression levels of all the FOX genes in the transcriptome microarray data derived from 30 patient-derived GSC samples as well as 4 normal astrocytes primary cultures (Fig. 2D). Among 51 FOX family members, FOXM1 exhibited strikingly higher expression in GSCs in comparison to normal astrocytes, unlike any other FOX family members. In all of the tested 10 GSC samples, we observed 6–12-fold higher FOXM1 expression by quantitative RT-PCR compared to astrocytes (Fig. 2E). CD133 expression on the cell surface is associated with cancers stem cells in some, if not all, GBM tumors [1, 4, 16, 32-34]. We further performed cell sorting by using CD133 marker and separated CD133 (+) and CD133 (−) cells for GBM157 and GBM528 GSCs. qRT-PCR demonstrated higher expression of FOXM1 and MELK in CD133 (+) cells than CD133 (−) cells in both samples (Fig. 2F).

We then examined FOXM1 protein expression in human GSCs. In agreement with previous studies, immunohistochemistry of a human GBM specimen demonstrated strong immunoreactivity for FOXM1 in the nuclei of GBM cells (Supporting Information Fig. S4) [35]. Immunocytochemistry of dissociated GBM NS demonstrated that FOXM1 was colocalized with the stem cell-associated markers including Nestin and SOX2 but not with the differentiation markers, TuJ1 or GFAP (Fig. 2G). Protein expression analysis by flow cytometry also yielded similar results. Similar to the higher proportion of tumor cells expressing the NSC-associated protein SOX2 and in contrast to the lower proportion of tumor cells expressing the astrocytic marker GFAP in GSCs in serum-free conditions, FOXM1 (+) cells were predominantly detected in GSCs in serum-free medium (GBM NSs), when compared to GBM cells propagated in serum-containing medium (SPGCs) (Fig. 2H; Supporting Information Fig. S5). Subsequently, we analyzed FOXM1 promoter activity in GSCs and non-GSCs in GBM30. GSCs manifested a significantly higher FOXM1 promoter activity in comparison to SPGCs (Supporting Information Fig. S6). Taken together, FOXM1 expression and activity are substantially elevated in GSCs compared to SPGCs and normal cells in mouse and human brains.

FOXM1 Is a Substrate for MELK

Prior studies have suggested that both FOXM1 and MELK play essential roles in the cell cycle progression, cancer cell growth, and maintenance of stem cell state of GBM [18, 20, 32, 35, 36]. Nonetheless, no studies have demonstrated a direct molecular interaction of MELK and FOXM1. We therefore sought to elucidate the potential molecular interaction of FOXM1 and MELK. First, we used the TCGA array data set (GEO database accession number GDS1815) to determine the similarities of the expression profiles between FOXM1 and MELK in high-grade glioma (Fig. 3A). Using 24 grade III glioma samples and 56 GBM samples, we found that FOXM1 RNA expression in individual tumors was correlated with MELK expression (p < .001). Significant correlation was also observed in newly diagnosed and recurrent GBM patient samples at RNA levels (Supporting Information Fig. S7). We then sought to expand our analysis of their expression in GBM tissues through The Cancer Genome Atlas (TCGA), a publicly available repository which has accumulated comparative genomic hybridization, gene expression, and DNA methylation analyses for 218 GBM samples [24]. Using this database, coexpression of MELK in GBM tumor samples was confirmed (p < .0001) with FOXM1 expression. Immunocytochemistry with patient-derived GBM NS (GBM30) exhibited colocalization of FOXM1 with MELK within individual cells (72.5% ± 2.4% colocalization in Fig. 3B).

Since FOXM1 and MELK are expressed in the same cells, we next investigated whether levels of MELK influence FOXM1 expression and vice versa (Fig. 3C; Supporting Information Fig. S8).
Both RT-PCR and Western blot demonstrated that neither MELK overexpression nor knockdown affected FOXM1 expression. We then asked whether FOXM1 physically interacts with MELK. HEK 293 cells overexpressing both Flag-tagged MELK and the fusion protein of FOXM1 and EGFP or EGFP alone (control) were subjected to EGFP trap, followed by Western blot with MELK or Flag antibody (Fig. 3D, upper panel; Supporting Information Fig. S9). Cells co-overexpressing MELK and FOXM1 exhibited a stronger band for MELK than the control samples, implying a physical interaction between MELK and FOXM1 protein. This protein-protein interaction is likely dependent on MELK kinase activity, as the band intensity for the co-immunoprecipitation (IP) of the catalytically dead MELK mutant (D150A) was substantially diminished.
MELK in an in vitro phosphorylation assay. In parallel, we used purified CDK2/cyclin A kinase, a known FOXM1 kinase, as a positive control [37]. The autoradiogram clearly shows in vitro phosphorylation of FOXM1 by MELK and CDK2/cyclinA. In addition, FOXM1 phosphorylation was not observed when incubated with kinase dead mutant MELK D150A. The same amount of FOXM1 was present in all conditions, as is shown on the corresponding Coomassie staining. Collectively, these data indicate that MELK directly interacts with FOXM1 and thereby, phosphorylates FOXM1 in a kinase dependent manner.

**Figure 3.** FOXM1 is a substrate for MELK. (A): Comparison of MELK and FOXM1 expression profile (Affymetrix Human Genome U133A Array) indicates statistically significant correlation of the expression of these two genes in grade III glioma (left) (n = 24) and in GBM (middle) (n = 56). The Cancer Genome Atlas (TCGA) analysis of MELK and FOXM1 expression profile in 218 GBM patient samples indicates statistical significance (right panel). (B): Representative images of immunocytochemistry with GBM30 neurospheres for FOXM1 (red), MELK (green), Hoechst dye for nuclear staining (blue). Original magnification: ×40. Scale bar = 20 μm. For quantification, GBM30 samples were used and the experiment was repeated four times. (C): Reverse transcription polymerase chain reaction (RT-PCR) analysis for MELK and FOXM1 expression in GBM neurospheres treated with siRNA targeting three different sequences for FOXM1 (left). RT-PCR analysis for MELK and FOXM1 expression in GBM neurospheres treated with different doses of siRNA targeting MELK (right) n = 5. (D): Upper panel: Overexpression of Empty EGFP (control) + MELK-Flag (Flag-MELK), EGFP-FOXM1 + MELK-Flag, or EGFP-FOXM1 + MELK D150A-Flag plasmids in HEK293 cells are processed to GFP-trap followed by immunoblotting with anti-Flag antibody. Middle panel: Autoradiogram displaying in vitro phosphorylation of FOXM1 by the kinase domain of MELK (1–340). Lane 1: FOXM1 + ATP-Mg (no kinase); lanes 2 and 6: FOXM1 + ATP-Mg + MELK (1–340); lane 3: FOXM1 + MELK (1–340) (no ATP-Mg); lane 4: FOXM1 + ATP-Mg + CDK2/CyclA; lane 5: FOXM1 + ATP-Mg + CDK2/CyclA + roscovitine; lane 7: FOXM1 + ATP-Mg + MELK1–340(D150A). Lower panels: Coomassie staining of the samples subjected to autoradiography. Abbreviations: GBM, glioblastoma multiforme; IP, immunoprecipitation.

**MELK Activates FOXM1 Transcriptional Activity Leading to Upregulation of Mitotic Gene Expression**

To understand more in depth the molecular mechanism of MELK-dependent FOXM1 signaling, we investigated whether MELK regulates FOXM1 transcriptional activity using the 6× FOXM1-TATA-luciferase reporter plasmid. Transgene expression of wild-type FOXM1 alone activated its transcriptional activity, suggesting self-activation in GBM30 GSCs (Fig. 4A). Transgenic expression of wild-type MELK significantly
enhanced FOXM1 self-activation in a concentration-dependent manner (Fig. 4A). On the other hand, transgenic expression of the kinase dead mutant MELK D150A eliminated FOXM1 self-activation in a concentration-dependent manner. Since FOXM1 is known to regulate the transcriptional network of genes essential for mitotic progression (e.g., Aurora B kinase, CENPA, Survivin, and CyclinB1), we examined whether the FOXM1-MELK complex had any effect on mitotic regulators in GBM30 GSCs. Cotransfection of FOXM1 with MELK resulted in a marked elevation of the key mitosis genes (Fig. 4).

Figure 4. MELK phosphorylates FOXM1 and regulates FOXM1 activity, leading to upregulation of mitotic gene expression. (A): Graph indicating the FOXM1 promoter activity in 293T cells transfected with the 6×FOXM1 TATA-luciferase plasmid together with expression vectors encoding wild-type FOXM1 and increasing amounts of plasmids encoding either WT MELK or kinase-dead mutant form of MELK (D150A). The experiment was performed in triplicate in 96 well plates and repeated three times independently. (B): Relative mRNA expression levels of Survivin, CyclinB1, CDC25B, and Aurora B by quantitative reverse transcription polymerase chain reaction (qRT-PCR) in GBM30 cells transfected with GFP, FOXM1, FOXM1 + WT MELK, and FOXM1 + MELK D150A mutant. qRT-PCR was performed in triplicate and repeated three times independently. Abbreviations: EGFP, enhanced green fluorescent protein; WT, wild type.
Siomycin A Abrogates FOXM1-MELK Interaction

Previously, the thiazole antibiotic Siomycin A was identified as a FOXM1 inhibitor by diminishing its protein and mRNA abundance, and we recently discovered that Siomycin A also reduced MELK expression in GSCs in vitro [9, 18, 40]. Consistent with these findings, flow cytometry demonstrated that Siomycin A reduced the proportions of FOXM1 (+) and MELK (+) cells in GBM NS in a dose-dependent manner (Fig. 6A). We then investigated the effect of Siomycin A on FOXM1 activity driven by FOXM1 and/or MELK in GBM30 NS forming cells of treated and control tumors. Tumors resulted in the formation of GBM-like tumors in immunocompromised mouse brains. Similar to human GBM, the xenograft-derived tumors exhibited massive intratumoral hemorrhage, necrosis, and densely packed tumor cells with hyperchromatic nuclei surrounding tumor vessels (Fig. 7B).

Combined Treatment of Temozolomide with Siomycin A on GSCs-Derived Mouse Tumors Yields Better Survival than Monotherapy with Temozolomide

Accumulated evidence suggests that GSCs are relatively resistant to Temozolomide chemotherapy partly due to elevated expression of the drug resistance genes compared to non-GSCs [42–45]. We found that Temozolomide treatment of GBM30 GSCs paradoxically increases the expression of the drug resistance genes compared to non-resistant to Temozolomide chemotherapy partly due to elevated expression of the drug resistance genes compared to non-GSCs [42–45]. We found that Temozolomide treatment of GBM30 GSCs paradoxically increases the expression of the drug resistance genes compared to non-resistant to Temozolomide chemotherapy partly due to elevated expression of the drug resistance genes compared to non-GSCs [42–45]. We found that Temozolomide treatment of GBM30 GSCs paradoxically increases the expression of the drug resistance genes compared to non-resistant to Temozolomide chemotherapy partly due to elevated expression of the drug resistance genes compared to non-GSCs [42–45]. 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Figure 5. MELK-driven FOXM1 phosphorylation is dependent on PLK-1. (A): Graph indicating the FOXM1 promoter activity in 293T cells with transfection of the FOXM1 reporter plasmid together with the plasmids encoding WT FOXM1 (FOXM1WT) and MELK WT, PLK-1 WT, or combination, in the presence of different doses (100, 250, and 500 nM) of the PLK-1 inhibitor ON-01910 for 48 hours. (B): Graph indicating the FOXM1 promoter activity in 293T cells with transfection of the FOXM1 reporter plasmid together with the plasmids encoding either FOXM1WT and plasmids encoding for WT or kinase-dead mutant (D150A) of MELK or PLK-1 WT and DN PLK-1 mutant. (C): Graph indicating the FOXM1 promoter activity in indicated cells with transfection of the FOXM1 reporter plasmid together with the plasmids encoding constitutive active mutant FOXM1 (FOXM1EE) and plasmids encoding for WT or kinase-dead mutant (D150A) of MELK. (D): Graph indicating the FOXM1 promoter activity in indicated cells with transfection of the FOXM1 reporter plasmid together with the plasmids encoding either WT FOXM1 (FOXM1WT) or FOXM1 mutants (715A, 724A, or 715/724A) and plasmids encoding for WT or kinase-dead mutant (D150A) of MELK. (E): Graph indicating the FOXM1 promoter activity in indicated cells with transfection of the FOXM1 reporter plasmid together with the plasmids encoding either WT FoxM1 (FOXM1WT) or FOXM1 mutants (S596A, S678A, or TSAA) and plasmids encoding for WT or kinase-dead mutant (D150A) of MELK. All luciferase experiments were performed using 96 well plate and repeated three times independently. Abbreviations: DN, dominant negative; GBM, glioblastoma multiforme; WT, wild type.
assayed at 2 days post-Siomycin A injection had significantly fewer NS-forming cells compared to the control tumors (Fig. 7D). These data suggest that Siomycin A has a potent inhibitory effect on survival and proliferation of GSCs in vivo. Subsequently, we assessed if Siomycin A treatment has any survival benefit on Temozolomide-treated mouse tumors. When the tumors were treated with Temozolomide alone, median survival of tumor-bearing mice was prolonged from 11.5 days to 22.5 days (Fig. 7E). The combined treatment with Temozolomide and Siomycin A exhibited further benefit on mouse survival (median survival to 28 days) (DMSO control vs. Temozolomide þ Siomycin A: p < .001).

Finally, we used the tumor slice culture method to assess Siomycin A treatment on surgical GBM specimens [46]. Patient-derived GBM slice cultures were treated with DMSO, Temozolomide, or Siomycin A, and the effects were measured 16 hours post-treatment. H&E staining of the slice cultures demonstrated that the procedures did not destroy the cytarchitecture of GBM tumors (Supporting Information Fig. S10). Immunohistochemistry with a proliferation marker, Ki67 demonstrated vast numbers of proliferating tumor cells in DMSO-treated samples, but not in Temozolomide- or Siomycin A-treated tissues (Supporting Information Fig. S11). In turn, immunohistochemistry for apoptosis marker activated Caspase-3 displayed significantly higher number of apoptotic cells in Siomycin A-treated samples, but not in DMSO- or Temozolomide-treated cultures (Supporting Information Fig. S11).

DISCUSSION

Molecular signaling between protein kinases and transcription factors plays vital roles in tumor development and maintenance [47]. Here, we demonstrate the first evidence that the transcription factor/oncogene FOXM1 forms a protein
complex with a serine/threonine kinase MELK and that FOXM1 serves as a substrate of MELK in cancer cells, and MELK-regulated FOXM1 phosphorylation controls FOXM1 activity and induces the expression of downstream mitotic regulators. The transcription factor FOXM1 is a master regulator for cell cycle progression and is overexpressed in a number of human cancers including GBM [48]. The protein kinase MELK is also abundantly expressed in various cancers including GBM and plays a pivotal role in survival of cancer cells and cancer stem cells [12]. Recent studies have shed light on FOXM1 signaling in GBM and GSCs [10]. FOXM1 interacts with the promoter of the VEGF gene regulating its activation, which contributes to GBM tumor angiogenesis [49]. Another study by Zhang et al. elegantly showed that FOXM1 is a downstream component of canonical Wnt-signaling and directly binds to β-catenin, inducing its nuclear translocation and transcriptional activation [39]. This study demonstrates the first evidence that FOXM1 directly interacts and is

Figure 7. Combined treatment of TMZ with SM on glioma stem-like cell (GSC)-derived mouse tumors yields better survival than monotherapy with TMZ. (A): Flow cytometry analysis for FOXM1 (upper panels) and MELK (lower panels) with GBM30 SPGCs (serum-propagated GBM cells) treated with varying doses of TMZ for 72 hours. Experiment was repeated three times for confirmation of results. (B): Representative images of mouse brains with intracranial xenograft tumors derived from GBM30 neurospheres (left top panel). Middle and lower pictures indicate H&E staining. N indicates necrotic area in the tumor. Original magnifications: ×2 (middle panel) and ×10 (lower panel). Scale bars = 500 μm (middle panel) and 100 μm (lower panel). (C): Ki-67 immunohistochemistry of mouse tumors treated with either DMSO or SM. Mice were sacrificed at 2 days post-SM treatment. Original magnifications: ×20. Scale bars = 50 μm. Graph (right) indicating the proportion of Ki67(+) cells in DMSO- and SM-injected tumors analyzed by Image J software. (n = 4 for each group) Asterisk (*) indicates statistical significance by t test. (D): Graph indicating the relative neurosphere numbers derived from mouse tumor tissues following DMSO and SM treatment for 2 days. (n = 3) Asterisk (*) indicates statistical significance by t test. (E): Kaplan Meier survival curve of mice harboring GBM30 neurosphere-derived tumors treated with DMSO (control), TMZ (10 mg/kg), or TMZ (10 mg/kg) combined with SM injection (2.5 nM). Table (right) indicates the mean and medial survival periods of the three groups. Abbreviations: DMSO, dimethyl sulfoxide; FITC, fluorescein isothiocyanate; FSC, forward scatter; SM, Siomycin A; TMZ, Temozolomide.
phosphorylated by MELK in GSCs, further providing evidence for a role of FOXM1 signaling in GBM. Our data indicate that both phosphorylation of FOXM1 protein and transcriptional activity of FOXM1 depend on MELK kinase activity. Intriguingly, FOXM1 activation driven by MELK is dependent on both the priming event of the complex formation of FOXM1 with PLK1 and the subsequent trans-activation of FOXM1 by PLK1.

Despite the advances presented here, open questions still remain. First, it is not clear whether the MELK/FOXM1 protein complex plays a positive role in cancers or cancer stem cells in other organs. Second, it is undetermined whether MELK-mediated signaling is associated with the other proteins that regulate, or are regulated by, FOXM1 in cancer cells. Future studies are needed to elucidate these questions.

The cell of origin for GBM is still debatable, although the concept has been evolving. Here, we used a mouse model of gliomagenesis by ablation of key tumor suppressor genes in NPCs causing formation of GBM-like tumors in cerebral cortices. Our data indicated that FOXM1 signaling is operative in NPCs of the developing brains in vivo. Immunohistochemistry exhibited that abundance of FOXM1 protein is progressively elevated as cells progress from the neural progenitor stage through pretumorigenic progenitors to GSC. Sensitivity of GSCs to Siomycin A was markedly higher than that of NPCs. Taken together, these data indicate that GSCs are more dependent on FOXM1 signaling compared to NPCs. However, it remains unknown whether activation of FOXM1 signaling alone is sufficient for gliomagenesis and whether FOXM1 signaling plays a critical role in oligodendrocyte precursor-derived gliomagenesis. Future studies could help address these questions.

An important therapeutic implication of the present data is that combined therapy of mouse xenografted tumors with Temozolomide and Siomycin A resulted in diminished NS-forming GSCs in tumors and provided an additional benefit on mouse survival. Although eradication of cancer stem cells appears to be essential for the cure of cancers, recent studies also suggest that non-cancer stem cells acquire cancer stem cell phenotype when challenged by stressors such as ionizing radiation and chemotherapies [50]. To achieve cure of a cancer, eradication of the rare population of tumor cells that are considered as existing cancer stem cells may not be sufficient; instead, combination of non-cancer stem cell-targeted therapies and cancer stem cell-targeted therapies appears to be mandatory. Our data indicate that Temozolomide treatment increased, while Siomycin A decreased, both FOXM1 and MELK expression in GSCs. One interpretation of these data is the preferential eradication of non-stem tumor cells and subsequent enrichment of GSCs after treatment (selection of therapy-resistant tumor cell population; the clonal evolution theory). Alternatively, Temozolomide may induce phenotypic changes of the treated GBM cells and may increase FOXM1 and MELK expression (the molecular evolution theory). It is also possible that both theories are true. Future studies will address this open question.

In this study, we demonstrated that the oncoprotein FOXM1 is regulated by the mitotic kinase MELK for its phosphorylation and autoactivation via a direct interaction in GSCs. Although FOXM1 is restrictedly expressed in NPCs in the normal brain and plays a role in NPC growth, a substantial elevation of FOXM1 during gliomagenesis and elevated sensitivity of GSCs over NPCs to pharmacological inhibition of FOXM1 signaling indicate that GSCs are more dependent on FOXM1 signaling for their survival and growth. Thus, targeting the ability of FOXM1 to form a protein complex with MELK may represent a potential therapeutic benefit for GBM. The results presented here may help to gain further insights into the biology of GSCs as well as molecular mechanisms of tumorigenesis and therapy resistance in GBM.

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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