MEF Promotes Stemness
in the Pathogenesis of Gliomas

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SUMMARY

High-grade gliomas are aggressive and uniformly fatal tumors, composed of a heterogeneous population of cells that include many with stem-cell-like properties. The acquisition of stem-like traits might contribute to glioma initiation, growth, and recurrence. Here we investigated the role of the transcription factor myeloid Elf-1 like factor (MEF, also known as ELF4) in gliomas. We found that MEF is highly expressed in both human and mouse glioblastomas and its absence impairs gliomagenesis in a PDGF-driven glioma mouse model. We show that modulation of MEF levels in both mouse neural stem cells and human glioblastoma cells has a significant impact on neurosphere formation. Moreover, we identify Sox2 as a direct downstream target of MEF. Taken together, our studies implicate MEF as a previously unrecognized gatekeeper gene in gliomagenesis that promotes stem cell characteristics through Sox2 activation.

INTRODUCTION

Malignant gliomas represent the most prevalent primary brain tumor in adults and inevitably have a poor prognosis. Despite the implementation of new therapeutic strategies, the median survival of patients with Glioblastoma multiforme (GBM), the most aggressive glioma variant, is only 14–16 months and these tumors remain rapidly and uniformly fatal (Wen and Kesari, 2008).

GBMs are very heterogeneous tumors that contain both neoplastic and nonneoplastic cells, including endothelial, stromal, and inflammatory cells (Charles et al., 2011). A fraction of cells within the tumor, identified as glioma stem-like cells (GSCs), share some common features with normal neural stem cells (NSCs); they are multipotent and have the property of self-renewal. These cells may either derive from adult undifferentiated stem and progenitor cells or could acquire stem-like properties as a result of the genetic alterations that promote the tumorigenic process (Holmberg et al., 2011). When implanted into the brain of immunodeficient animals, these GSCs are capable of generating new tumors at high efficiency (Galli et al., 2004). Moreover, GSCs are remarkably resistant to the chemotherapy and radiotherapy (Dean et al., 2005) used as standard first-line treatment of patients with malignant gliomas. Indeed, acquisition of stem-like characteristics likely contributes to the malignant nature of high-grade gliomas and may be responsible for the initiation, growth, and recurrence of these tumors.

Myeloid Elf-1 like factor (MEF, also known as ELF4) is a member of the ETS family of transcription factors, which contains over 30 family members. Several ETS proteins can function as oncogenes and show aberrant expression in solid tumors as well as in hematological malignancies (Sashida et al., 2010). While MEF has been proposed to function as a tumor suppressor gene in some contexts, it could contribute to tumor formation in mice as well as in humans (Mikkers et al., 2002) (Sashida et al., 2010). Insight into its mechanism of action has come from studies done in fibroblasts demonstrating MEF's ability to induce transformation by stimulating Mdm2 expression, thereby downregulating p53-dependent responses, and by inhibiting activation of Ink4a, thereby allowing unrestrained phosphorylation of the retinoblastoma (Rb) protein (Sashida et al., 2009). Some of the ETS proteins are known to play
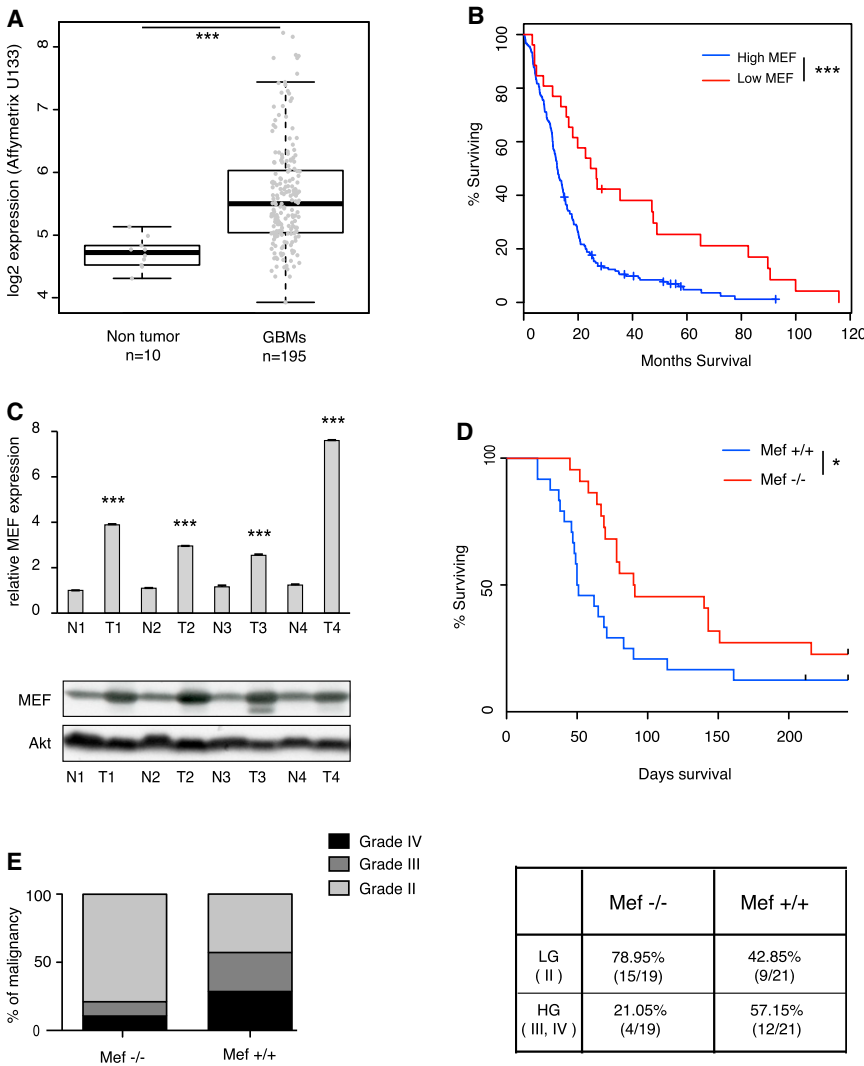
Figure 1. MEF in Human and Mouse Gliomas

(A) MEF expression in the TCGA data set obtained from human GBM samples (n = 195) and nontumor brain tissue (n = 10) (***p = $2.366e^{-09}$, Student's t test).

(B) Kaplan Meier survival curves of TCGA GBM patients: low level of MEF (calculated as less than one standard deviation from the mean of diploid tumors) significantly correlates with better overall survival (***log-rank p value, p = 0.000386).

(C) Top panel: MEF mRNA level detected by RT PCR is higher in PDGF-driven mouse GBMs (T) compared to normal tissue (N). Data are normalized to GAPDH expression. Results are presented as mean ± SD (***p < 0.0001, Student's t test). Bottom panel: western blot showing that MEF protein level is higher in PDGF-driven mouse GBMs compared to normal tissue.

(D) Kaplan Meier survival curves of PDGF gliomas generated in Ntv-a $Mef^{+/+}$ versus $Mef^{-/-}$ mice. MEF loss increases overall survival and reduces gliomagenesis (log-rank p value, *p = 0.02).

(E) Left panel: mouse gliomas lacking MEF show significantly lower percentage of high-grade versus low-grade tumors (Fisher's exact test, *p = 0.027); right panel: contingency table presenting the number of high-grade (HG) tumors versus low-grade (LG) tumors in the two different genetic backgrounds.

See also Figure S1.

a role in brain tumors (Uht et al., 2007), though so far no published data are available regarding the role of MEF in normal brain or in brain tumor biology. Here we show that MEF is highly expressed in GBMs and it contributes to gliomagenesis by promoting stem cell traits through direct activation of Sox2 expression.

RESULTS

MEF Is Highly Expressed in Human Gliomas and Its Loss Impairs Glioma Formation in Mice

GBM represents a heterogeneous disease and recent genomic analyses have quantified the expression level of a wide variety of genes (Cancer Genome Atlas Research Network, 2008). We analyzed the data set from The Cancer Genome Atlas (TCGA) for GBMs and found MEF expression significantly elevated in the tumor samples (n = 195) as compared to nontumor brain tissue (n = 10) (p < 0.0001; Student's t test) (Figure 1A and Table S1 available online), without evidence for amplification at the genomic level. Because MEF has previously been shown to block the p53 pathway, we examined the p53 status of these

tumors and found no correlation between MEF expression levels and p53 mutation or deletion, suggesting that MEF may function in a p53-independent manner in glioma (Figure S1A available online).

A search conducted in the Oncomine website (https://www.oncomine.org//) revealed that in the Sun data set (Sun et al., 2006), which includes 81 human GBMs (grade IV), 25 anaplastic astrocytomas (grade III), and 50 oligodendrogliomas (grade II), MEF expression is significantly higher in GBMs than in lower grade gliomas (p < 0.0001) (Figure S1B). To confirm these data we analyzed MEF expression by qPCR in 25 human glioma surgical samples from Memorial Sloan-Kettering Cancer Center (MSKCC) (six oligodendrogliomas, seven anaplastic oligodendrogliomas, two anaplastic astrocytomas, and ten glioblastomas) and found that MEF is significantly elevated in high-grade versus low-grade gliomas (p = 0.02, Student's t test) (Figure S1C). However, this difference might reflect a lower ratio of tumor cells versus nontumor cells in the low-grade samples.

Lower levels of MEF were associated with a better prognosis in a small cohort of acute myeloid leukemia patients (Fukushima et al., 2003). We examined the TCGA GBM patient survival data and found that low levels of MEF significantly correlated with better overall survival in GBMs as well (log rank test, p = 0.0008; Figure 1B). Gene expression profiling studies have identified four molecular subclasses of GBMs based on transcriptional signatures: Classical, Mesenchymal, Proneural and Neural. Each of these subtypes has been associated with
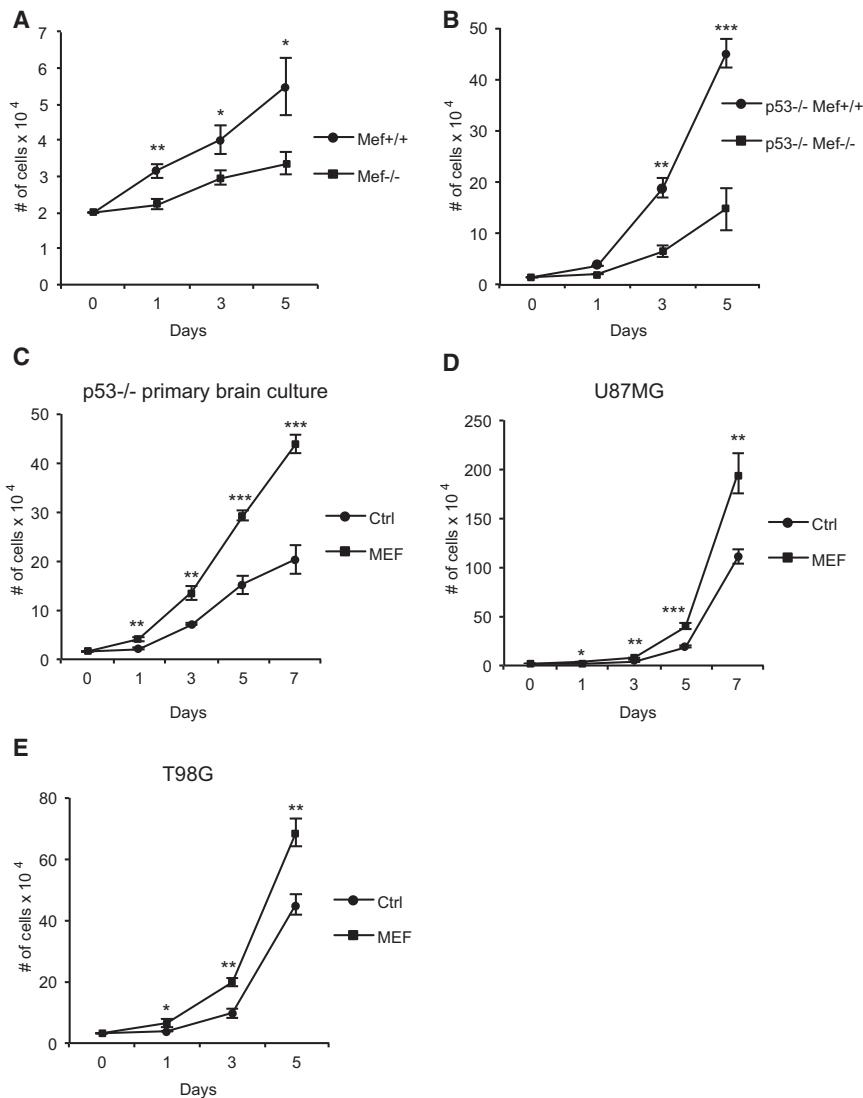
Figure 2. Mef Promotes Proliferation of both Primary Brain Cultures and Human Glioma Cell Lines

(A and B) Growth curve of primary brain cultures derived from $Mef^{-/-}$, $Mef^{+/+}$ (A) and $p53^{-/-}/Mef^{-/-}$, $p53^{-/-}/Mef^{+/+}$ (B) newborn mice.

(C–E) Growth curve of primary brain cells derived from $p53^{-/-}$ newborn mice (C) and human U87MG and T98G glioma cell lines (D and E).

Results are presented as mean ± SD from a representative of three experiments performed in triplicate. *p < 0.05; **p < 0.001; ***p < 0.0001 (Student's t test). See also Figure S2.

Ink4a/Arf null background, in which PDGF is able to uniformly generate high-grade gliomas that share hallmark histological features with human GBMs (Dai et al., 2001). Using qPCR and Western blot analysis, we found higher levels of MEF mRNA and protein in these tumors as compared to the contralateral normal brain tissues (Figure 1C).

Next, to determine if lack of MEF impacts on PDGF-induced gliomagenesis, $Mef^{-/-}$ mice were crossed with Ntv-a mice (which carry a wild-type Ink4a/Arf locus) to generate Ntv-a $Mef^{+/+}$ and Ntv-a $Mef^{-/-}$ mice. A survival analysis of the two cohorts revealed that loss of MEF significantly impaired PDGF-induced glioma formation, with MEF null mice living an average of 129 days (n = 22) and wild-type mice living 59 days (n = 24) (p < 0.02, log rank test) (Figure 1D). The tumors were then scored and graded by histological features (see Experimental Procedures for details). Tumors lacking MEF showed less aggressive features, with significantly fewer high-grade gliomas (p = 0.02, Fisher's exact test) (Figure 1E). Thus, MEF can affect both glioma formation and progression.

MEF Promotes Proliferation of both Mouse Primary Brain Cultures and Human Glioma Cell Lines

As MEF has been shown to promote the transition of cells from G1 to S (Liu et al., 2006; Sashida et al., 2009) we evaluated its effect on proliferation, using both mouse primary brain cultures and human glioma cell lines. The primary cultures were generated from the whole brain of newborn pups, and to investigate potential p53-independent effects, we used cells from $Mef^{+/+}$ and $Mef^{-/-}$ mice and also from $p53^{-/-}/Mef^{+/+}$ and $p53^{-/-}/Mef^{-/-}$ mice. Cells lacking MEF grew more slowly than the control cells (Figures 2A and 2B), while its overexpression in $p53^{-/-}$ cells (Figure S2A) increased proliferation (Figure 2C). Similarly, the overexpression of MEF in the human U87MG and T98G glioma cell lines (Figures S2B and S2C) resulted in higher rates of cell proliferation (Figures 2D and 2E).

specific signaling alterations, such as EGFR, Ras, and PDGFR pathway activation, respectively (Verhaak et al., 2010). When the patients were stratified according to GBM subtypes, the Proneural subclass showed the lowest level of MEF expression (Figure S1D) and, within this subtype, lower levels of MEF also correlated with better overall survival (Figure S1E). When we looked at the IDH1 status, we found that the tumors with the lowest MEF levels within the Proneural group were predominantly IDH1 mutant tumors, whereas IDH1 wild-type Proneural tumors had a range of MEF levels similar to the other tumor types (data not shown).

To determine whether MEF plays an active role in gliomagenesis, we used the RCAS/PDGF mouse glioma model, which closely resembles the human Proneural GBM subtype. The RCAS/tv-a system utilizes avian leukosis virus based vectors (RCAS) to mediate gene transfer into somatic cells, engineered to be transgenic for its receptor (tv-a). Specifically, we used Nestin tv-a (Ntv-a) mice, where the tv-a receptor is under the control of the Nestin promoter, a well-known marker of progenitor and neural/glial cells. First, we evaluated MEF expression in an

These data confirm the role of MEF in promoting the growth of both mouse primary brain cultures and human glioma cell lines.

MEF Promotes Stem Cell Characteristics in Mouse NSCs and Human Glioma Cell Lines

In recent years, an increasing number of studies have investigated the connection between malignancy and "stemness," focusing on how stem/progenitors cells, as well as neoplastic cells, change their properties during the process of malignant transformation. Several transcription factors are known to induce pluripotent stem cells from differentiated cells, as well as to maintain multipotency of NSCs (Patel and Yang, 2010). Because MEF contributes to glioma formation and aggressiveness in vivo, we explored the possible role of MEF in promoting stem cell characteristics.

NSCs and GSCs can be grown as spheres in culture, in the absence of serum but in the presence of basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF). Although sphere-forming assays cannot be considered an exact readout of in vivo stem cell activity, they are useful to measure the in vitro potential of cells to exhibit stem-cell-like traits (Pastrana et al., 2011); therefore, we investigated whether MEF could influence the formation of neurospheres.

Freshly isolated $Mef^{-/-}$ and $Mef^{+/+}$ cells from postnatal non-neoplastic brains were plated at different cell densities (10, 5, and 1 cells/μl) in 24-well plates and grown in neurosphere medium for 2 weeks to allow the generation of neurospheres. We then performed serial passages over the course of 8 weeks, generating secondary, tertiary, and quaternary spheres. Spheres were mechanically dissociated every 2 weeks, and at each passage, sphere number was assessed. We found that at a density of 1 cell/μl, lack of MEF significantly decreased generation of secondary, tertiary, and quaternary neurospheres (Student's t test, p < 0.0001, at each passage) (Figure 3A). Similar results were obtained at higher cell concentrations (5 and 10 cells/μl) (Figure S3A). Moreover, cells lacking MEF underwent morphological changes over time, with some of them becoming attached to the bottom of the wells and presenting short elongation processes (Figure S3B). To understand whether the decreased sphere forming ability seen in $Mef^{-/-}$ cells was accompanied by increased differentiation, we grew cells from dissociated spheres in 5% serum medium without FGF and EGF to evaluate their differentiation potential. Phase contrast images revealed clear differences in the morphology of the $Mef^{+/+}$ and $Mef^{-/-}$ cells, suggesting that differentiation is more profound in cells lacking MEF (Figure 3B). Immunofluorescence staining showed a significant reduction in Nestin+ cells (p = 0.0006), together with a significant increase in both Olig2 and GFAP+ cells (p < 000.1 and p = 0.0481, respectively, Student's t test) in $Mef^{-/-}$ cells (Figures 3C and 3D). These differences were detected also by Western blot analysis (Figure 3E). Interestingly, most of the GFAP-positive $Mef^{-/-}$ cells showed the stellate morphology typical of astrocytes and a brighter signal, which was confirmed by increased GFAP protein levels measured by Western blot (Figure 3E). We failed to detect Tuj-1+ cells in three of the four $Mef^{-/-}$ samples, while an average of 14.4% Tuj-1+ cells was found in four different $Mef^{+/+}$ samples (data not shown), which suggests that MEF might also contribute to differentiation toward the neuronal lineage.

To determine whether lack of MEF could influence the stem cell potential under more strict conditions, we generated primary cell cultures obtained from the whole brain of newborn pups, forced them to grow in serum for two passages, and switched them to neurosphere medium. To evaluate possible p53-independent effects, primary brain cultures were isolated from different genetic backgrounds ($Mef^{+/+}$, $Mef^{-/-}$, $p53^{-/-}/Mef^{+/+}$, and $p53^{-/-}/Mef^{-/-}$). Four days after the switch to neurosphere medium, cells lacking MEF failed to form spheres, while both the $Mef^{+/+}$ and the $p53^{-/-}/Mef^{+/+}$ cells were able to do so (p = 0.0003 and p = 0.0001, respectively, Student's t test) (Figure 3F). The impaired neurosphere formation of the $p53^{-/-}/Mef^{-/-}$ cells could be rescued by MEF re-expression (p = 0.00145, Student's t test) (Figure 3G and Figure S3C).

We then investigated whether MEF overexpression could induce reprogramming signals under the same strict conditions, influencing the sphere-forming ability of different primary and glioma cells. We first overexpressed MEF in primary brain cultures obtained from p53 null pups, using retroviral vectors (Figure S2A). After antibiotic selection, the cells were grown in serum for two passages and then switched to neurosphere medium. We found that cells overexpressing MEF formed more neurospheres than the control cells (p = 0.0012 at 24 hr, p = 0.0017 at 72 hr; Student's t test) (Figure 3H). Similar differences upon MEF overexpression were observed in the U87MG and T98G human glioma cell lines (p < 000.1 and p < 000.1, respectively, Student's t test) (Figure 3I).

Lastly, we evaluated the impact of MEF on stem-like properties using human primary GSCs, with various genetic profiles. Tumor samples, isolated from patients suffering from GBMs and undergoing surgery at MSKCC, were dissociated and initially grown in neurosphere medium as a monolayer on plastic cell culture dishes coated with 10 ng/ml laminin. Specifically, we used the following cells: GBM1 (++PDGFRA, +EGFR, MET, CDK6, −PTEN, −CDKN2A), GBM3 (++EGFR, +MET, CDK6, −CDKN2A,PTEN, carrying EGFRvIII mutation), and GBM543 (++PDGFRA, ++CDK4, +(EGFR,MET,CDK6), −PTEN) (Ozawa et al., 2010; Pulvirenti et al., 2011). Using lentiviral vectors to express shRNAs, we silenced MEF expression (Figure S3D) and found that decreased MEF levels lead to a significant decrease in neurosphere formation in all of these three GSC lines (p < 0.0001 for both shRNA#1 and shRNA#2), as compared to nontargeting shRNA control (Figure 3J). Moreover, when we performed limiting dilution neurosphere assays, we confirmed that MEF knockdown leads to a reduced frequency of sphere-forming cells (p < 000.1 for both shRNAs in each GSC line, ELDA software) (Figure 3K).

Taken together, these data suggest a role for MEF in promoting stem-cell-like features in both primary brain cultures and glioma cell lines.

Sox2 Gene Is a Direct Target of MEF and Rescues Sphere-Forming Ability in MEF-Defective Cells

To investigate possible mechanisms behind the ability of MEF to promote stem-cell traits, we measured the expression level of several genes related to pluripotency, including Sox2, Oct4, Nanog, Klf4, Hes1, and Hey1, in MEF-transduced $p53^{-/-}$ primary brain cells. Among these genes, we found that Sox2 and Oct4 expression was significantly increased by MEF. We
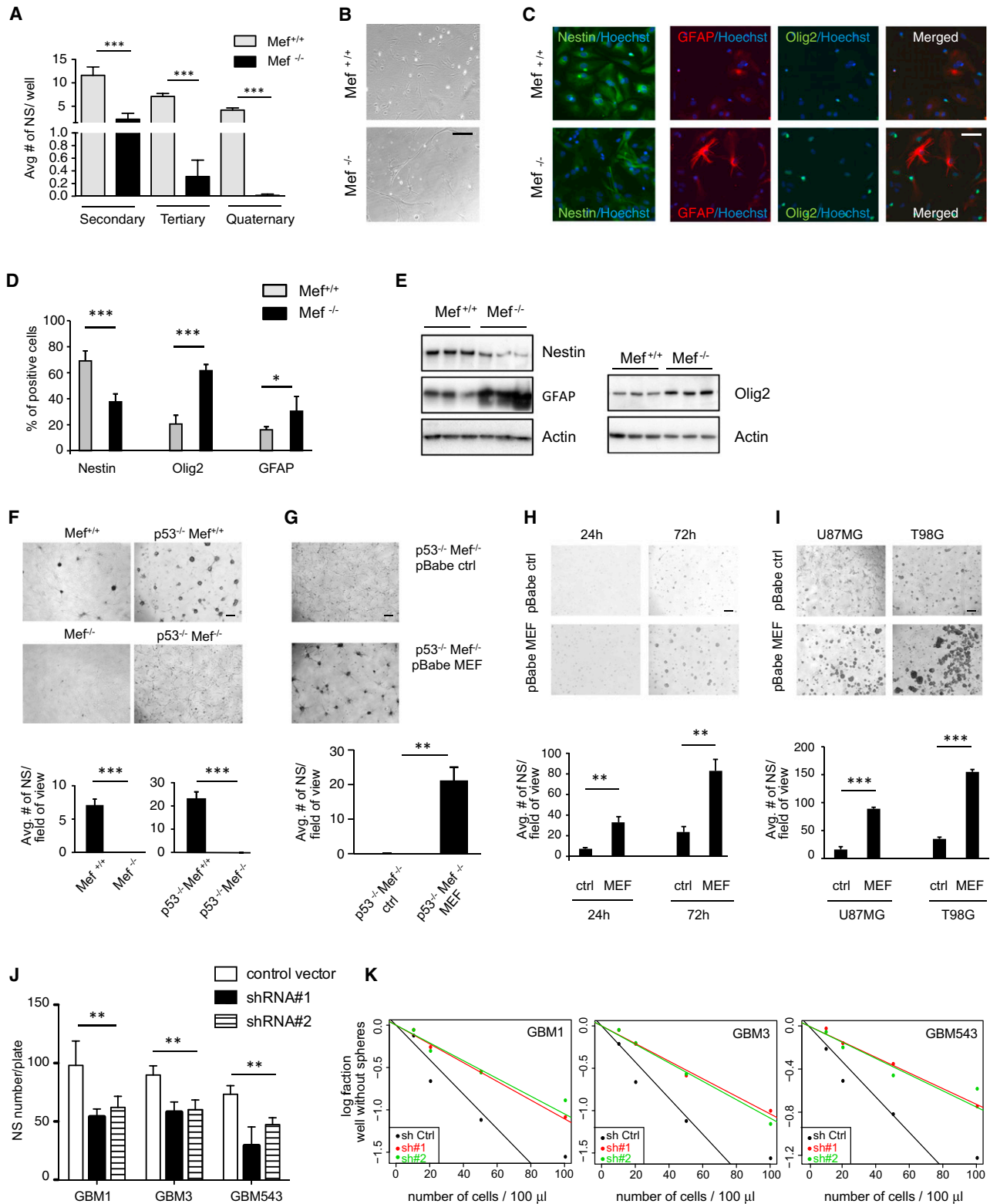
Figure 3. MEF Promotes Stem Cell Traits of both Primary Brain Cultures and Glioma Cell Lines

(A) Neurospheres isolated from newborn pups, with $Mef^{+/+}$ and $Mef^{-/-}$ genetic background, respectively: MEF loss led to impaired neurosphere formation after serial passages.

(B) Phase contrast images showing morphological changes between $Mef^{+/+}$ and $Mef^{-/-}$ brain cells grown in 5% serum, without bFGF and EGF, for 5 days to favor differentiation.

observed a 3.6-fold increase in Sox2 gene expression and a 3-fold increase in Oct4 expression level (p = 0.02 and p = 0.013, respectively, Student's t test) (Figure 4A). Similarly, in U87MG and T98G cells, MEF induced a 2.1 (p = 0.017) and 1.6 (p = 0.018) -fold increase in Sox2 levels and a 1.8 (p = 0.0025) and 1.6 (p = 0.0199) -fold increase in Oct4 levels (Figure 4B).

The Sox2 promoter is known to contain ETS binding sites (Wiebe et al., 2000), though they have not been identified in the Oct4 promoter; therefore, we focused on Sox2 as a possible direct downstream target of MEF. We analyzed Sox2 expression in $Mef^{+/+}$ versus $Mef^{-/-}$ and $p53^{-/-}/Mef^{+/+}$ versus $p53^{-/-}/Mef^{-/-}$ primary brain cells and found a significant reduction in Sox2 levels in the absence of MEF (p < 0.0001, Student's t test) (Figures 4C and 4D). To exclude the possibility that developmental adaptation to the lack of MEF resulted in decreased Sox2 expression, we acutely knocked down MEF in p53 null primary brain cultures (∼60%) and found a significant reduction in Sox2 expression (p = 0.015 and p = 0.0059, respectively, Student's t test) (Figure 4E). Thus, both the acute loss and chronic absence of MEF substantially decreases Sox2 expression.

To test whether MEF can activate the Sox2 promoter, we transiently transfected a Sox2 promoter-driven luciferase reporter plasmid (Kuwabara et al., 2004) into p53 null primary brain cells, together with MEF or an empty vector control. As shown in Figure 4F, MEF induced a greater than 3-fold increase in luciferase activity from the Sox2 promoter (p < 0.0001, Student's t test) while having no effect on Hes1 promoter-driven luciferase activity. We also investigated whether MEF activates the Sox2 promoter in human glioma cell lines and found a similar upregulation (p < 0.0001, Student's t test) (Figures 4G and 4H).

Having identified Sox2 as a transcriptional target of MEF, we investigated whether Sox2 overexpression could rescue the decrease in neurosphere formation seen in cells lacking MEF. We overexpressed Sox2 in $p53^{-/-}/Mef^{-/-}$ primary brain cultures (Figure S4B) and grew them in neurosphere conditions. The absence of neurospheres seen in cells lacking MEF was rescued by Sox2 overexpression (p = 0.00018, Student's t test), indicating that Sox2 acts downstream of MEF to promote neurosphere formation (Figure 4I).

To confirm that Sox2 gene regulation by MEF was direct, we performed Chromatin Immunoprecipitation (ChIP) assays using T98G glioma cells that were transduced with a pBabe retroviral vector expressing a FLAG-tagged MEF cDNA or a control vector. Using an anti-FLAG ChIP assay, we found that in human glioma cells, MEF protein is recruited to specific regions of the Sox2 gene (which covered −1.964, −1.672, and −1.324 kb from the transcription starting site [TSS]; p values: 0.0009, 0.003, 0.0008, respectively). No direct binding was observed at the farthest region (−3.841 kb) (Figure 4J).

DISCUSSION

The regulation of stemness is of interest to many disciplines, including developmental biology, regenerative medicine, degenerative disease, and cancer. Normal stem cells are known to play critical roles in tissue development, differentiation, and organogenesis (Vierbuchen et al., 2010). Several transcription factors, such as Notch, Id1, Sox2, Oct4, and others, have been reported to control different aspects of stemness, influencing the delicate balance between stem cell maintenance and the promotion of stem cell differentiation. In recent years, many studies have shown that genes involved in normal stem cell biology also play a relevant role in tumorigenesis (Nam and Ben-ezra, 2009) (Venere et al., 2011).

Malignant gliomas are highly aggressive cancers composed of a heterogeneous cell population, a fraction of which exhibits stem-like characteristics (Vescovi et al., 2006). Whether GSCs arise from normal NSCs or from neoplastic cells that have acquired stem cell traits is still unclear; however, it has been proposed that the acquisition of stem cell characteristics through the activation of stem cell gene signatures can confer malignant potential to gliomas (Holmberg et al., 2011).

We have shown that MEF, a member of the ETS family of transcription factors, contributes to gliomagenesis and promotes stem-like characteristics. MEF is highly expressed in both human and mouse GBMs, and GBM patients with low levels of MEF show a significantly better overall survival. This finding was replicated in the RCAS/PDGF model where gliomas that lack MEF had less aggressive histological features and better overall survival.

Genes overexpressed in cancer can affect many different biological processes including growth, stress response, block of apoptosis, and stemness. Uncontrolled proliferation is a key aspect of cancer cell behavior and we found that MEF promotes the proliferation of both mouse primary brain cultures and human glioma cell lines, contributing to the pathogenesis of gliomas.

(C and D) Immunofluorescence staining and quantification of $Mef^{+/+}$ and $Mef^{-/-}$ brain cells grown in the above condition to favor differentiation. Positive staining is quantified as percentages of total Hoechst+ cells per image (10 fields of images were taken from four different cell lines for each genotype, $Mef^{-/-}$ and $Mef^{+/+}$); scale bars indicate 50 μm.

(E) Western blot comparing $Mef^{-/-}$ to $Mef^{+/+}$ brain cells: lack of MEF lead to decreased Nestin and increased Olig2 and GFAP protein levels.

(F) $Mef^{+/+}$, $Mef^{-/-}$, $p53^{-/-}/Mef^{+/+}$, and $p53^{-/-}/Mef^{-/-}$ primary brain cultures grown in neurosphere conditions. Scale bars indicate 100 μm.

(G) Re-expression of MEF in $p53^{-/-}Mef^{-/-}$ cells rescues their ability to form neurospheres when cultured in neurosphere conditions.

(H) Micrograph showing $p53^{-/-}$ primary brain cells transfected with pBabe-empty and pBabe-MEF retroviral vectors, cultured in neurosphere conditions. Bar graphs on the bottom show average neurosphere number per field of view.

(I) MEF overexpressing human U87MG and T98G cell lines, cultured in neurosphere conditions, form more neurospheres compared to control. Bar graphs on the bottom show average neurosphere number per field of view.

(J) Graph bar showing decreased neurosphere formation after MEF knockdown in patient-derived human primary GBMs (GBM1, GBM3, and GBM543).

(K) Graph representing limiting dilution neurosphere assays in patient-derived human primary GBMs (GBM1, GBM3, and GBM543) infected with pGipz control vector and pGipz MEF shRNAs #1 and #2.

Results are presented as mean ± SD from a representative of three experiments performed in triplicate, unless otherwise specified. *p < 0.05; **p < 0.005; ***p < 0.0001 (Student's t test). See also Figure S3.
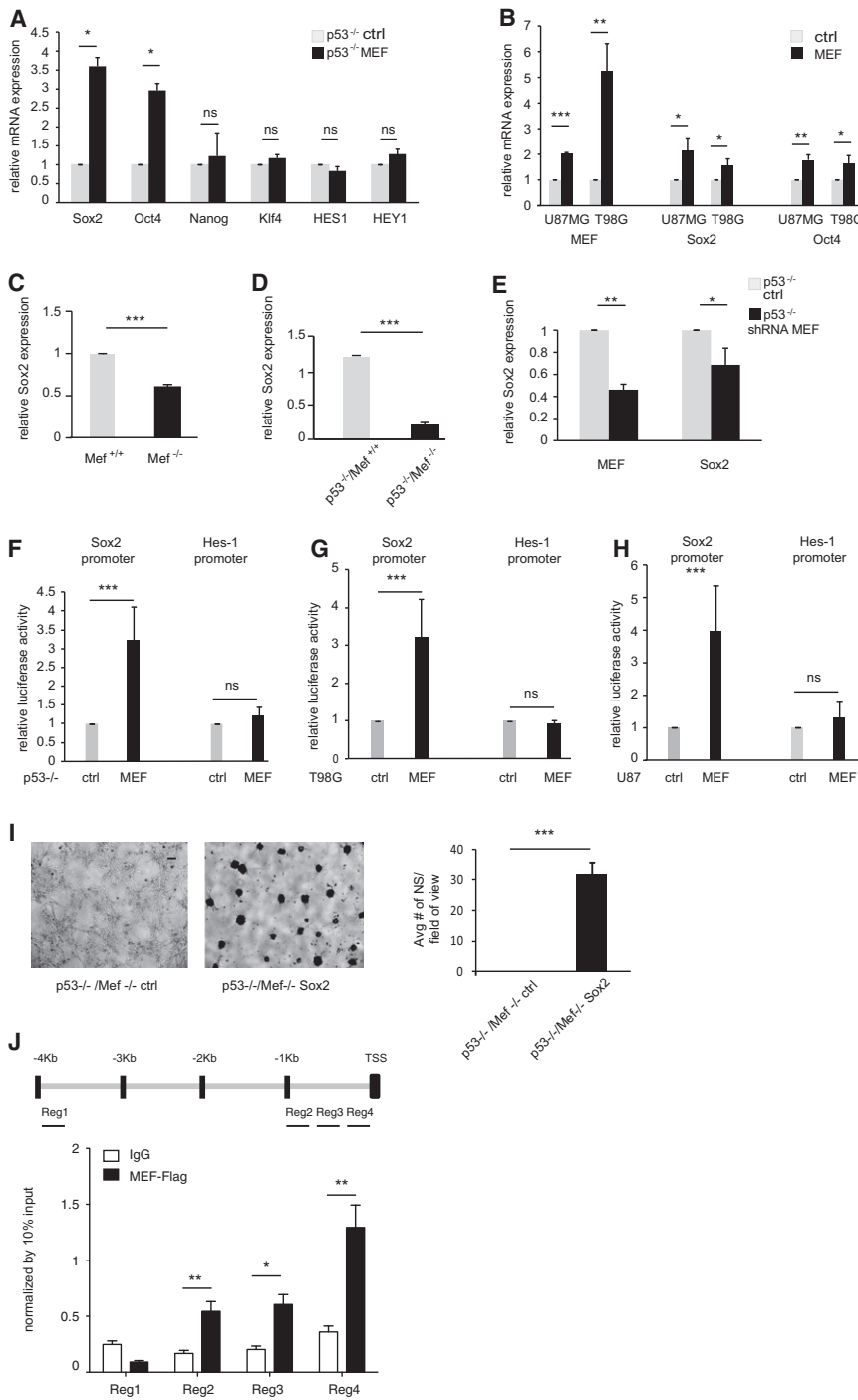
Figure 4. Sox2 Gene Is Regulated by MEF

(A and B) MEF overexpression in $p53^{-/-}$ primary brain cells as well as in U87MG and T98G human glioma cell lines led to increased Sox2 expression. Data are normalized to GAPDH expression.

(C and D) Lack of MEF decreases Sox2 expression. Data are normalized to GAPDH expression.

(E) Acute knockdown of MEF in $p53^{-/-}$ primary brain cells led to decreased Sox2 expression. Data are normalized to GAPDH expression.

(F–H) Dual luciferase assays showing that MEF activates Sox2 promoter in $p53^{-/-}$ primary brain cells as well as T98G and U87MG human glioma cell lines.

(I) Sox2 overexpression in $p53^{-/-}/Mef^{-/-}$ primary brain cultures grown in neurosphere conditions rescues their ability to form neurospheres. Scale bar indicates 100 μm. Bar graph shows average neurosphere number per field of view.

(J) ChIP experiments were performed on T98G glioma cells using antibody to Flag. Plotted values are relative enrichments (y axis) to 10% input and measured for sites in the Sox2 promoter (x axis). Antibody against IgG was used as a nonspecific control.

Results are presented as mean ± SD from a representative of three experiments performed in triplicate. *p < 0.05; **p < 0.001; ***p < 0.0001 (Student's t test). See also Figure S4.

which might reflect changes in the stem cell signature of both neoplastic and non-neoplastic cells.

We have further implicated MEF in promoting stem cell traits through direct activation of Sox2 expression. The HMG-box transcription factor Sox2 is known to play an important role in maintaining stem cell self-renewal within the central nervous system (CNS) and this activity is present in gliomas as well (Pevny and Nicolis, 2010). Sox2 has been described to act as an oncogene in different human cancers including gliomas. While the role of Sox2 in normal brain and glioma cells has been well documented, the transcription factors that activate its expression in these contexts are less clear (Denysenko et al., 2010; Ikushima et al., 2009). We identified Sox2 as a direct target of MEF in mouse primary brain cells and human glioma cell lines, because both acute and chronic modulation of MEF levels affected Sox2 gene expression and promoter activation. MEF directly binds to Sox2 promoter in T98G glioma cells. We observed that overexpression of Sox2 in $p53^{-/-}/Mef^{-/-}$ cells was sufficient to rescue the impaired ability of these cells to form neurospheres. Thus, our data suggest that Sox2 is downstream of MEF and may be responsible for its ability to modulate stem-like characteristics.

Given the cellular heterogeneity and molecular complexity of gliomas, it is unclear in which contexts the stem cell property

Another key feature of glioma malignancy is the activation of neural and pluripotent stem cell gene expression signatures. We found that mouse primary brain cells that lack MEF and silencing of MEF in human primary glioblastoma stem-like cells each lead to an impaired capacity to generate spheres, together with an increased differentiation potential. Conversely, MEF overexpression increases neurosphere formation of both mouse primary brain cells and glioma cell lines. Collectively, our data suggest that MEF plays a role in promoting stem-like traits,

of self-renewal might represent a feature associated with increased tumorigenic potential (Barrett et al., 2012). Additionally, the so called "glioma stem-like cells" within different tumors may vary in terms of proliferation rates, which might impact their aggressiveness regardless of their self-renewal properties. To date, it is often difficult to distinguish between the contributions of proliferation versus self-renewal to the process of gliomagenesis; nonetheless, self-renewal inevitably entails proliferative events. Adding to the intricacy, acquisition of reprogramming signals generated by alterations in specific genes or pathways (Notch, Id1, Sox2, Oct4, etc.) might differentially impact on these two diverse aspects.

Our work defines the role of MEF in promoting stem cell features in both primary mouse brain cells and human glioblastoma cells via direct regulation of Sox2 expression, providing insights into the multifaceted regulation of stemness. Further work is necessary to better define the MEF/Sox2-mediated acquisition of stem cell traits and whether it modulates the response to chemotherapy and radiotherapy.

EXPERIMENTAL PROCEDURES

TCGA Analysis
TCGA data was downloaded from the TCGA Data Portal (https://tcga-data.nci.nih.gov/) or the cBio Cancer Genomics Portal (Cerami et al., 2012) (http://cbioportal.org/). Subtype information was retrieved from Verhaak et al. (2010). Low MEF expression in the TCGA data set was defined using the median expression data (median of three platforms, see Verhaak et al., 2010) as one standard deviation lower than the mean of all tumors. All other analyses were performed using data from the Affymetrix U133 microarray platform. Expression data was available for 195 tumors and 10 normal brain samples (Table S1).

Generation of Primary Brain Cultures and Primary Brain Neurosphere and Neurosphere Formation Assay
The $Mef^{+/+}$, $Mef^{-/-}$, $p53^{-/-}$, and $p53^{-/-}Mef^{-/-}$ primary brain cultures were prepared with mechanical dissociation of the whole brain from newborn mice. The cells were then filtered through a 70 μm Nylon strainer and plated in 10 cm culture dishes (with the procedure derived with minor modifications from a previously described experimental method) (Dai et al., 2001). Mouse primary brain cultures, as well as T98G and U87MG human glioma cell lines, were plated at 20 cells/μl in neurosphere medium. Mouse primary brain neurospheres were isolated as previously described (Bleau et al., 2009) and plated at different concentrations (10, 5, and 1 cell/μl). Human primary glioblastoma neurospheres were freshly isolated from patients suffering from GBMs and undergoing surgery at MSKCC. Primary GBM samples were dissociated, plated at different concentrations (1, 0.5, 0.2, and 0.1 cells/μl) and grown in neurosphere medium. Neurosphere medium consisted of NSC Basal Medium, NSC proliferation supplements, 10 ng/ml EGF, 20 ng/ml basic-FGF, and 1 mg/ml Heparin (Stem Cell Technologies, Vancouver, Canada). Neurosphere number was established by counting the number of neurospheres per well or, for U87MG and T98G, by taking pictures of the central part of the plates and then counting the neurosphere number per field of view.

SUPPLEMENTAL INFORMATION

Supplemental Information for this article includes four figures, Supplemental Experimental Procedures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.stem.2012.09.012.

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