MIF Maintains the Tumorigenic Capacity of Brain Tumor–Initiating Cells by Directly Inhibiting p53

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Abstract

Tumor-initiating cells thought to drive brain cancer are embedded in a complex heterogeneous histology. In this study, we isolated primary cells from 21 human brain tumor specimens to establish cell lines with high tumorigenic potential and to identify the molecules enabling this capability. The morphology, sphere-forming ability upon expansion, and differential potential of all cell lines were indistinguishable in vitro. However, testing for tumorigenicity revealed two distinct cell types, brain tumor–initiating cells (BTIC) and non-BTIC. We found that macrophage migration inhibitory factor (MIF) was highly expressed in BTIC compared with non-BTIC. MIF bound directly to both wild-type and mutant p53 but regulated p53-dependent cell growth by different mechanisms, depending on glioma cell line and p53 status. MIF physically interacted with wild-type p53 in the nucleus and inhibited its transcription-dependent functions. In contrast, MIF bound to mutant p53 in the cytoplasm and abrogated transcription-independent induction of apoptosis. Furthermore, MIF knockdown inhibited BTIC-induced tumor formation in a mouse xenograft model, leading to increased overall survival. Collectively, our findings suggest that MIF regulates BTIC function through direct, intracellular inhibition of p53, shedding light on the molecular mechanisms underlying the tumorigenicity of certain malignant brain cells. Cancer Res; 76(9); 2813–23. ©2016 AACR.

Introduction

Glioblastoma, the most common form of primary malignant brain tumor, is nearly always fatal (1). Although neurosurgeons may be able to remove the main tumor mass, this procedure typically leaves behind a small population of tumor cells that retain the capacity for tumorigenesis (2). For this reason, glioblastomas almost always relapse, highlighting the need for more precisely targeted therapeutic approaches. A number of recent studies have demonstrated that tumors are composed of cells with functional heterogeneity that form a hierarchy, especially in the context of tumor initiation (3, 4). Characterized by its heterogeneous tumor types (5–8), glioblastoma is now also thought to be initiated and maintained by a subpopulation of tumor cells, called brain tumor stem cells or brain tumor-initiating cells (BTIC; refs. 9–13). The clinical significance of BTICs is emphasized by the strongly enhanced capacity of these cells to initiate brain tumors, in contrast to the majority of tumor cells, which may contribute significantly to the tumor mass, but not to the initiation and maintenance of the tumor. BTICs are thus considered a promising target for future therapies.

Macrophage migration inhibitory factor (MIF) was originally identified as a proinflammatory cytokine, but was later found to be a critical regulator of tumor progression as well (14–18). MIF upregulation has been observed in human melanoma (19), lung cancer (20), prostate cancer (21), hepatocellular carcinoma (22), and glioblastoma (23–27). MIF expression was significantly higher in the tumor tissues of glioblastoma patients than in normal brain tissues (24, 25). The precise roles of MIF in the initiation and progression of glioma, however, remain unclear.

MIF plays numerous roles through receptor-mediated signaling pathways that are activated by cell surface receptors, such as CD74, CD44, and CXCRs (28–30), or by intracellular interactions through binding to JAB1 (31). MIF has also been shown to function as a p53 inhibitor (16) that directly binds and antagonizes p53 function in the cells (32).

The p53 protein is an important and well-characterized tumor suppressor (33). In response to various forms of cell stress, p53 regulates diverse target molecules, which may induce cell-cycle arrest and apoptosis in a transcription-dependent manner (34). p53 has also been shown to possess other biologic activities, including induction of apoptosis, in mitochondria in a transcription-independent manner (35–37).

In this study, we show that MIF expression is significantly higher in BTICs isolated from human glioma tissues than in non-BTICs from the same tissue source and that MIF acts as a direct intracellular p53 inactivator that regulates cell proliferation and apoptosis in glioma cells. In addition, we highlight the link between the roles of MIF and tumor-initiating cells associated with p53, a candidate for the dedifferentiation barrier.
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Table 1. Isolation of BTICs

<table>
<thead>
<tr>
<th>Tumor type</th>
<th>Age (y)</th>
<th>Sex</th>
<th>Culture</th>
<th>Differentiation potential</th>
<th>Inoculation of the tumor cells</th>
<th>In vivo tumor formation</th>
<th>PS3</th>
</tr>
</thead>
<tbody>
<tr>
<td>hG002 Pilocytic astrocytoma</td>
<td>29</td>
<td>M</td>
<td>Impossible</td>
<td>+</td>
<td>Tested</td>
<td>-</td>
<td>M</td>
</tr>
<tr>
<td>hG006 Anaplastic astrocytoma</td>
<td>43</td>
<td>M</td>
<td>Temporarily</td>
<td>+</td>
<td>Tested</td>
<td>-</td>
<td>M</td>
</tr>
<tr>
<td>hG001 Glioblastoma</td>
<td>46</td>
<td>M</td>
<td>Temporarily</td>
<td>Not tested</td>
<td>Not tested</td>
<td>-</td>
<td>M</td>
</tr>
<tr>
<td>hG004 Glioblastoma</td>
<td>64</td>
<td>F</td>
<td>Temporarily</td>
<td>+</td>
<td>Not tested</td>
<td>-</td>
<td>M</td>
</tr>
<tr>
<td>hG006 Glioblastoma</td>
<td>25</td>
<td>F</td>
<td>Impossible</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>M</td>
</tr>
<tr>
<td>hG008 Glioblastoma</td>
<td>61</td>
<td>F</td>
<td>Permanently</td>
<td>+</td>
<td>Tested</td>
<td>+</td>
<td>M</td>
</tr>
<tr>
<td>hG010 Glioblastoma</td>
<td>50</td>
<td>F</td>
<td>Temporarily</td>
<td>+</td>
<td>Tested</td>
<td>-</td>
<td>M</td>
</tr>
<tr>
<td>hG011 Glioblastoma (Gliosarcoma)</td>
<td>40</td>
<td>M</td>
<td>Temporarily</td>
<td>+</td>
<td>Tested</td>
<td>-</td>
<td>M</td>
</tr>
<tr>
<td>hG003 Glioblastoma</td>
<td>39</td>
<td>F</td>
<td>Temporarily</td>
<td>+</td>
<td>Tested</td>
<td>-</td>
<td>M</td>
</tr>
<tr>
<td>hG014 Glioblastoma</td>
<td>37</td>
<td>F</td>
<td>Temporarily</td>
<td>+</td>
<td>Tested</td>
<td>-</td>
<td>M</td>
</tr>
<tr>
<td>hG015 Glioblastoma</td>
<td>81</td>
<td>F</td>
<td>Temporarily</td>
<td>+</td>
<td>Tested</td>
<td>-</td>
<td>M</td>
</tr>
<tr>
<td>hG017 Glioblastoma</td>
<td>37</td>
<td>F</td>
<td>Temporarily</td>
<td>+</td>
<td>Tested</td>
<td>-</td>
<td>M</td>
</tr>
<tr>
<td>hG018 Glioblastoma</td>
<td>65</td>
<td>F</td>
<td>Temporarily</td>
<td>+</td>
<td>Tested</td>
<td>-</td>
<td>M</td>
</tr>
<tr>
<td>hG020 Glioblastoma</td>
<td>53</td>
<td>M</td>
<td>Permanently</td>
<td>+</td>
<td>Tested</td>
<td>+</td>
<td>WT</td>
</tr>
<tr>
<td>hG021 Glioblastoma</td>
<td>73</td>
<td>M</td>
<td>Impossible</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>M</td>
</tr>
<tr>
<td>hG007 Oligodendroglioma</td>
<td>30</td>
<td>M</td>
<td>Temporarily</td>
<td>+</td>
<td>Tested</td>
<td>-</td>
<td>M</td>
</tr>
<tr>
<td>hG005 Anaplastic oligodendroglioma</td>
<td>81</td>
<td>F</td>
<td>Temporarily</td>
<td>Not tested</td>
<td>-</td>
<td>-</td>
<td>M</td>
</tr>
<tr>
<td>hG002 Anaplastic oligodendroglioma</td>
<td>36</td>
<td>F</td>
<td>Temporarily</td>
<td>+</td>
<td>Tested</td>
<td>-</td>
<td>M</td>
</tr>
<tr>
<td>hG019 Anaplastic oligodendroglioma</td>
<td>41</td>
<td>M</td>
<td>Permanently</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>WT</td>
</tr>
<tr>
<td>hG003 Medulloblastoma</td>
<td>34</td>
<td>M</td>
<td>Temporarily</td>
<td>+</td>
<td>Tested</td>
<td>-</td>
<td>M</td>
</tr>
<tr>
<td>hG009 Olfactory neuroblastoma</td>
<td>54</td>
<td>M</td>
<td>Impossible</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>M</td>
</tr>
</tbody>
</table>

Abbreviations: M, mutant; WT, wild-type; +, possess the potential; −, none of the potential.

Materials and Methods

Human tissues and cell culture

Approval to use human neurosphere cultures was obtained from the Keio University ethics committee. Tissue procurement procedures were performed in accordance with the Declaration of Helsinki and in compliance with the ethical guidelines of the Japan Society of Obstetrics and Gynecology and the Network of European CNS Transplantation and Restoration. Brain tumor samples were obtained from patients undergoing surgery at Keio University Hospital (Tokyo, Japan) following approval from the Institutional Review Board at Keio University School of Medicine (Tokyo, Japan). Tumor samples were mechanically and enzymatically dissociated into single cells. All tumor-derived cells and neural stem cells (NSC) were cultured at 37°C in a humidified incubator with 5% CO₂ in neurosphere medium (NSM) consisting of Neurobasal Medium (Invitrogen), B27 (Invitrogen), human recombinant bFGF and EGF (PeproTech, 20 ng/mL each), and heparin (10 ng/mL; Sigma). U87MG and T98G cells were provided by Dr. Y. Ohashi (Keio University) and maintained in DMEM supplemented with 10% FBS. Short tandem repeat DNA profiling of T98G and U87MG cells was performed using the Cell ID System (Promega) in August 2011. Human astrocytes were obtained from Lonza and maintained in Astrocyte Basal Medium (Lonza).

Animal models

For the brain tumor initiation assay, 6- to 8-week-old female NOD/SCID mice were purchased from Sankyo Lab. Samples of 1,000, 10,000, or 100,000 primary cells cultured from brain tumor tissues were stereotactically injected into the brain striatum (2.5 mm right of the midline, 3 mm deep). The mice were killed when they exhibited neurologic symptoms or at 150 days after inoculation, whichever came first. All procedures involving animal experiments were approved by the Animal Care and Use Committee of the Keio University School of Medicine (Tokyo, Japan). To evaluate the tumor-initiating potential of BTICs by treatment of lentivirus expressing MIF shRNA, two groups of mice were prepared. hG008 cells were infected with lentivirus expressing either MIF shRNA or control shRNA [multiplicity of infection (MOI) = 1] for 24 hours and washed twice in PBS, after which the number of live cells was counted. A total of 10,000 cells were inoculated into the brain striatum (n = 5); the mice were killed and tumor generation was evaluated by histologic examinations six weeks after implantation. For the treatment study using lentivirus, intracranial transplantation of BTICs was performed as described above. To establish glioma xenografts for lentivirus-mediated MIF shRNA treatment, hG008 cells were injected into the brains of NOD/SCID mice (3,000 cells/mouse, eight mice/group). Lentivirus expressing either MIF shRNA or shRNA control (MOI = 1) was injected twice into the tumor sites at two and three weeks after tumor cell implantation. The effects of MIF shRNA treatment were evaluated to compare the survival times of each group by Kaplan–Meier analysis.

Immunoprecipitation assay

Immunoprecipitation was performed using an nProtein A-Sepharose 4 Fast Flow Kit (GE Healthcare) following the manufacturer’s instructions. Nuclear and cytoplasmic extracts of tumor cells (1 mg) were suspended in 1 mL RIPA buffer (Pierce) and coupled to 50 µL nProtein A-Sepharose (GE Healthcare) for 1 hour at 4°C for precleaning. The supernatant was incubated with mouse anti-p53 (DO-1; 1:200; Santa Cruz Biotechnology) or nonspecific mouse IgG (sc-2025; Santa Cruz Biotechnology) antibody for 4 hours at 4°C. A total of 50 µL nProtein A-Sepharose was added to the samples, which were incubated for 1 hour at 4°C. The immune complexes were washed twice with RIPA buffer and once with wash buffer (50 mmol/L Tris, pH 8.0). The beads were resuspended in the sample buffer (1% SDS, 100 mmol/L DTT, 50 mmol/L Tris, pH 7.5) and boiled for 5 minutes before the supernatants were subjected to Western blot analysis.

Gel shift assay

Gel shift assays were performed using a P53 EMSA Kit (Panomics) following the manufacturer’s instructions. In each
sample, 10 μg of protein from nuclear extracts isolated from U87MG cells was incubated with biotinylated p53 consensus binding sequence oligonucleotides (AY1032P; Panomics). Separation of bound and free probe was achieved by electrophoresis using a 6.0% nondenaturing polyacrylamide gel, and the protein–DNA complexes were transferred to a nylon membrane (Hybond-N, GE Healthcare). The DNA was crosslinked to the membrane using a UV cross-linker. Protein–DNA complexes were detected using streptavidin–horseradish peroxidase and visualized after exposure to Hyperfilm ECL film (Amersham Biosciences). Experiments were performed independently at least twice.

Statistical analysis

Statistical analyses were performed using Statcel2. Student t tests were performed to determine the statistical significance of the quantitative PCR study, cell proliferation study, double knockdown study, reporter gene assay, and apoptosis assay. Mann–Whitney tests were used to assess in vivo tumor initiation after treatment with shRNAs. P < 0.05 was considered to represent significance.

All other methods are available in Supplementary Material and Methods.

Results

Isolation and characterization of BTICs

Twenty-one brain tumors were dissociated into single cells and plated at a clonal density (38) in NSM. These brain tumors and their characteristics are shown in Table 1. Four of the tumors, including two glioblastomas, one pilocytic astrocytoma, and one olfactory neuroblastoma, could not be cultured in NSM. Most of the primary cells could be cultured temporarily as glioma spheres but did not culture for more than 8 passages. Only three cultures, hG008, hG019, and hG020, could be cultured for more than 20 passages and proliferate permanently. These three cell lines included two glioblastomas, hG008 and hG020, and one anaplastic oligodendroglioma, hG019. Notably, hG008 and hG019...
were obtained from tumors that had recurred after surgery, radiation, and chemotherapy. hG020 was isolated from a patient who had undergone surgery for a newly diagnosed glioblastoma. Multiple point mutations in the DNA-binding domain of the p53 gene were found in hG008 (Supplementary Table S1). Wild-type p53 was expressed in hG019 and hG020. BTICs usually show potential for tumorigenesis, proliferation, self-renewal, and multidifferentiation (39). These three expandable primary cells generated glioma spheres and expressed nestin, a marker for NSCs (Fig. 1A). To assess their multilineage differentiation potential, the primary cells were cultured in differentiation medium containing 1% FBS without EGF and FGF2. All of the analyzed primary cells, including BTICs and non-BTIC, were able to differentiate into cells of three neural lineages: glial (GFAP), neuronal (β-III tubulin), and oligodendroglial (O4; Fig. 1B; Table 1).

Next, to determine whether the primary cells were tumorigenic in vivo, the primary cells were xenografted into the striatum of NOD/SCID mice. We attempted to generate tumors in the mouse brain by inoculating 1,000, 10,000, or 100,000 primary cells and observed tumorigenesis solely from hG008, hG019, and hG020 cells (Fig. 1C; Table 1), from which we concluded that hG008, hG019, and hG020 were BTICs. The other primary cells, which did not generate brain tumors in vivo but showed multilineage differentiation potential and limited potential for expansion in vitro, were defined as non-BTICs.

**BTICs expressed MIF and p53**

To identify molecules involved in brain tumorigenesis, we analyzed gene expression in BTICs and non-BTICs. MIF is a cancer-related gene that has previously been shown to promote cell survival and proliferation in mouse NSCs (40). Intriguingly, the MIF (Fig. 2A) gene was more highly expressed in BTICs than in non-BTICs, astrocytes, and NSCs. Next, MIF expression in human glioblastoma tissues was assessed by immunohistochemical analysis. Some tumor cells expressed MIF in both the cytoplasm and the nucleus (Fig. 2B). MIF was highly expressed in BTICs and glioblastoma tissues, although the gene expression level of CD74 and CXCR4, known as MIF direct binding receptors, was lower in cultured BTICs than in NSCs (Supplementary Fig. S1). We decided to focus on the intracellular functions of MIF in this study. MIF expression was further investigated in the cytoplasm and nucleus by immunoblotting (Fig. 2C). MIF protein expression was verified...
in the cytoplasmic and nucleic fractions in BTICs and glioblastoma cell lines (T98G, U87MG). Intriguingly, MIF expression was low in the cytoplasm and was not detected in the nuclei of NSCs and astrocytes. MIF has previously been identified as a molecule that inhibits p53 function (16), so we investigated p53 expression. The cytoplasmic fractions of hGO08, hGO19, and T98G displayed p53 expression, whereas hGO20, NSCs, and U87MG showed very low levels of p53 expression. In the nuclear fractions, p53 expression was high in BTICs and T98G and moderate in NSCs, astrocytes, and U87MG (Fig. 2C).

MIF binds to p53 and inhibits p53 function

We first examined the role of MIF in two glioblastoma cell lines, U87MG and T98G. Both cell lines expressed MIF and p53, but these exhibit different p53 gene mutation statuses: U87MG express wild-type p53, and T98G has a homozygous mutation at codon 237 (M→I) of p53 (41). MIF gene expression was knocked down using MIF siRNA in U87MG, and cell proliferation was assessed. U87MG cells treated with MIF siRNA showed decreased cell proliferation compared with siRNA controls (Fig. 3A). Next, we tested the role of p53 in this system. A p53 knockdown system was established using p53 siRNA in U87MG and T98G cells (Supplementary Fig. S2), and we found that p53 gene silencing abrogated the decrease in cell proliferation induced by MIF gene silencing in U87MG cells (Fig. 3B and Supplementary Fig. S2). This suggests a strong functional relationship between MIF and p53. We next explored the physical interaction between MIF and p53 in U87MG cells. Immunoprecipitation analysis was performed using U87MG nuclear protein, and we confirmed that p53 binds directly to MIF in the nuclei of U87MG (Fig. 3C). To investigate the regulation of p53 target genes under conditions in which MIF expression is suppressed, reporter gene assays were performed using p21-Luc and Bax-Luc reporter plasmids. The luciferase activities of p21 and BAX were elevated by MIF siRNA treatment compared with controls in U87MG (Fig. 3D). Immunoblot studies also confirmed upregulation of BAX and P21 protein in a dose-dependent manner by MIF siRNA treatment in U87MG cells (Fig. 3E). Cell-cycle analysis was performed by flow cytometry and G1 cell-cycle arrest was observed in U87MG in response to MIF siRNA treatment (Fig. 3F). In addition, a caspase-3/7 activity assay showed a dose-dependent increase in apoptosis following MIF siRNA treatment (Fig. 3G). Taken together, these results indicate that MIF downregulation induces cell-cycle arrest and apoptosis in U87MG cells.

To elucidate the functional relationship between p53 and MIF, changes in MIF-regulated p53 DNA-binding ability was assessed by gel shift assay. A p21 promoter DNA probe containing a p53-binding region was used in a gel shift assay using nuclear protein isolated from U87MG cells transfected with either MIF siRNA or control siRNA. The gel shift assay showed that p53-specific binding was upregulated by MIF siRNA compared with the control U87MG cells (Fig. 3H). MIF has thus been shown to bind to p53 in the nucleus of U87MG cells, and MIF siRNA-mediated gene silencing increased p53 DNA-binding ability, cell-cycle arrest, and apoptosis. We also assessed MIF function in T98G cells in the same manner as performed in U87MG cells and observed that MIF gene silencing by siRNA treatment decreased cell proliferation compared with the controls in a dose-dependent fashion (Fig. 4A and B). This reduction in cell proliferation was also p53 dependent in T98 cells, as seen in U87MG cells (Fig. 4C and Supplementary Fig. S2). However, in contrast to the results from U87MG cells, we did not observe an increase in the transcriptional activity of the p21 and BAX promoters following MIF siRNA treatment in the reporter gene assay, suggesting that mutated p53 does not activate the transcription of target genes in T98G cells (Supplementary Fig. S3A).

We therefore focused on the function of p53 in the cytoplasmic fraction of T98G cells and observed that p53 binds directly to MIF in this fraction (Fig. 4D). MIF siRNA treatment led to the induction of caspase-3/7 activity (Fig. 4E) without the induction of cell-cycle arrest in T98G cells (Supplementary Fig. S3B). We hypothesized that the decrease in cell proliferation induced by MIF gene silencing in T98G is regulated by proapoptotic events induced by p53 in the cytoplasmic fraction. In fact, MIF gene silencing led to the accumulation of p53 in the mitochondria fraction (Fig. 4F), which may lead to increased apoptosis in mitochondria. Moreover, treatment with PFT-μ, a known inhibitor of p53 translocation to mitochondria, abrogated the suppression in proliferation induced by MIF gene silencing in T98G cells (data not shown). These findings underscore the importance of MIF-dependent/induced p53 translocation to the mitochondria, which induces apoptosis in T98G cells. Collectively, these results show that MIF is able to bind to both wild-type and mutant p53, and to support cellular proliferation in a p53-dependent manner in both U87MG and T98G cells, although the underlying mechanisms differ between the two glioma cell lines.

MIF is the therapeutic target for BTICs

To confirm the effects of MIF on proliferation and apoptosis in BTICs, a lentivirus expressing MIF shRNA (lenti-shMIF) was employed, and the efficacy of the lentivirus was assessed by immunoblot analysis in hGO08 cells, which confirmed that changes had occurred in p53 protein expression (Fig. 5A). We evaluated the effects of lenti-shMIF on cell proliferation in three BTIC lines (hGO08, hGO19, and hGO20). Dose-dependent suppression of cell proliferation was observed in all BTICs on lenti-shMIF treatment (Fig. 5B). Immunoblot studies confirmed that MIF gene silencing led to the accumulation of p53 in the cytoplasmic fraction. In fact, MIF gene silencing led to the accumulation of p53 in the mitochondria fraction (Fig. 5C). Suppression of cell proliferation by MIF was also supported by p53 in hGO08 cells (Supplementary Fig. S4).

We further analyzed the gene expression change of p21 and BAX, known as transcription-dependent downstream targets of p53, in two BTICs (hGO08, p53 mutant-type; hGO20, p53 wild-type). U87MG (p53 mutant-type), and T98G (p53 mutant-type) to investigate the effect of MIF knockout. p21 and BAX genes were upregulated on infection with lenti-shMIF in hGO20 and U87MG compared with hGO08 and T98G in quantitative PCR study (Supplementary Fig. S5A and S5B). We also confirmed that MIF gene silencing led to the accumulation of p53 in the mitochondria fraction in hGO08, similar to the effect in T98G (Supplementary Fig. S5C). These results suggested that MIF inhibits the function of p53 in transcription-dependent and -independent manner, based on the status of the p53 gene in glioma cells and BTICs, respectively. Lenti-shMIF treatment of hGO08 and hGO20 cells also led to an increase in caspase-3/7 activity in a dose-dependent manner (Supplementary Fig. S5D).

We next examined the therapeutic effect of MIF targeting using mouse xenograft models. First, 1 × 10^4 hGO08 cells infected with either lenti-shMIF or lenti-shControl were implanted into the brains of immunodeficient NOD/SCID mice, and tumorigenicity was evaluated in each group at 42 days after implantation. The
Figure 3. MIF binds to p53 in the nucleus and inhibits p53 function in a transcription-dependent manner in U87MG cells. A, proliferation of U87MG cells was suppressed by MIF siRNA (siMIF) treatment compared with the controls (sicont.; mean ± SD). * P < 0.01; ** P < 0.001. B, the ability of MIF siRNA knockdown to suppress cell proliferation was abrogated by p53 gene silencing in U87MG cells at 7 days after transfection (mean ± SD). * P < 0.05. C, MIF binds to p53 in the nucleic fraction of U87MG cells. (Continued on the following page.)
implantation of hG008 cells infected with the control virus led to brain tumor formation in 4 of 5 mice, whereas no tumors were observed in mice implanted with lenti-shMIF–infected hG008 cells (n = 5), suggesting that MIF may be an essential factor in maintenance for brain tumors in this experimental system (Fig. 5D). Next, we attempted to validate the therapeutic effect of MIF gene silencing in BTICs. First, 1 × 10^6 hG008 cells were implanted in the brains of NOD/SCID mice and then a lentiviral vector expressing either MIF-shRNA or control-shRNA was administered to the BTIC implantation site on days 7 and 14 (Fig. 4).

**Figure 4.**
MIF binds to p53 in the cytoplasmic fraction of T98G cells and inhibits the apoptotic function of p53. A, decreases in MIF protein expression by gene silencing did not affect p53 protein expression at 3 days after MIF siRNA (siMIF) transfection. B, cell proliferation was suppressed by MIF siRNA transfection in a dose-dependent manner (mean ± SD). *, P < 0.05; ***, P < 0.001. C, the ability of MIF siRNA to suppress cell proliferation was abrogated by p53 gene silencing at 7 days after transfection (mean ± SD). ***, P < 0.001. D, MIF physically bound to p53 in the cytoplasmic fraction of T98G cells. E, MIF gene silencing in T98G cells led to an increase in caspase-3/7 activity compared with that of U87MG cells. 3 days after MIF siRNA transfection (mean ± SD). ***, P < 0.001. F, quantitation of p53 protein in mitochondria fraction by Western blot (WB) analysis showed increased p53 protein localization in the mitochondria of T98G cells following MIF gene silencing by infection with a lentivirus expressing MIF shRNA at 3 days after infection (mean ± SD). ***, P < 0.001. IP, immunoprecipitate.

(Continued) D, the transcriptional activities of cell-cycle regulator p21 and apoptotic factor BAX were elevated by MIF gene silencing at 3 days after transfection (mean ± SD). ***, P < 0.001. E, BAX and p21 protein expression was induced by MIF gene silencing in U87MG cells at 3 days after siRNA transfection. G, caspase-3/7 activity was increased at 3 days after MIF siRNA transfection in a dose-dependent manner (mean ± SD). ***, P < 0.001. H, the physical binding between a p53-specific DNA probe and nuclear extracts from U87MG cells was increased by MIF gene silencing. These physical interactions were antagonized by a cold probe and an anti-p53 antibody. The nuclear extracts were prepared from U87MG cells transfected with different siRNA concentrations at 3 days after transfection. IP, immunoprecipitate; LA, luciferase; WB, Western blotting.
Figure 5. MIF regulates the initiation of tumorigenesis in BTICs, and MIF gene silencing shows a therapeutic effect on BTICs in a xenograft model. A, hG008 cells infected with lenti-shMIF showed a significant decrease in MIF protein expression compared with controls (lenti-shControl) without affecting p53 protein expression at 3 days after infection. B, BTICs infected with lenti-shMIF inhibited cell proliferation in a virus dose-dependent manner at 7 days after infection (mean ± SD). *P < 0.05; **P < 0.01; ***P < 0.001. C, hG008 cells infected with lenti-shMIF did not form tumor spheres, whereas control cells grew while forming healthy tumor spheres. Scale bar, 200 μm. D, hG008 cells infected with lenti-shMIF did not generate brain tumors in mice, whereas 4 of 5 mice injected with hG008 cells infected with control virus generated brain tumors at 42 days after cell implantation (mean ± SD; P = 0.01). E, the brain tumor-bearing mice implanted with hG008 cells were infected with either lenti-shMIF (n = 8) or shControl (n = 8) virus at two and three weeks after cell implantation. Mice treated with lenti-shMIF showed longer survival compared with control mice, as evaluated by Kaplan-Meier analysis (mean ± SD; P = 0.04).
postimplantation. Survival periods were evaluated for each therapeutic group, and the mice inoculated with lent-shMIF lived significantly longer than the control mice (n = 8/group, P = 0.04; Fig. 5E and Supplementary Fig. S6). These results suggest that MIF plays a key role in the proliferation and tumorigenesis of BTICs, both in vitro and in vivo.

Discussion

High-viability brain tumor cells are known to be present in the restricted ring–enhanced region surrounding the tumor mass on gadolinium-enhanced MRI (42). Indeed, the efficiency of isolation of BTICs from glioma tissues is known to be quite low. One study showed that nine cell lines established from 19 brain tumor tissues could be cultured for more than eight passages as tumor spheres and that only six of the nine cell lines were transplantable into mouse brains for examination of their tumorigenic potential (43). In this study, we identified only three BTICs from 21 brain tumor masses, which we have compared in the current study to non-BTICs that showed no capacity for tumorigenicity. Previous BTIC studies have distinguished primary tumor cell types based on their expression of molecular markers, such as CD133. There are, however, no previous studies that distinguish BTICs and non-BTICs based on their functional properties. Therefore, the identification of high MIF expression in BTICs is important to gain a better understanding of the mechanisms underlying tumorigenesis.

We first investigated the functions of MIF in two glioblastoma cell lines, U87MG and T98G, as these were available for most reproducible examinations, have well-known biologic properties, including the status of the p53 gene, and also exhibit tumor-initiating potential.

We found high levels of MIF expression in the nuclei and cytoplasm of human glioma cells, but low expression of CD74, a direct binding receptor of MIF (29). A previous study reported scant expression of CD74 in several glioma cells (44). On the basis of the intracellular distribution of MIF in glioma cells, we focused on physical interactions between MIF and p53. Similar to the distinction observed the mutation status of p53, the intracellular distribution of p53 differed between U87MG and T98G cells. p53 was expressed in both the nucleus and cytoplasm of T98G cells, but only in the nucleus of U87MG cells. Our findings suggest that MIF exerts its functions through association with p53 mainly in the nuclear fraction of U87MG cells in a transcription-dependent manner. In T98G, MIF may inhibit transcription-independent apoptosis by binding to cytoplasmic p53, as the mutant p53 lacks transcription-activating ability (Supplementary Fig. S7). It has recently been reported that p53 exerts various biologic functions, including involvement in mitochondrial apoptosis in a transcription-independent fashion (35–37). In most cells with p53 mutations, including T98G and Hg008 cells, point mutations are located in the DNA-binding domain of p53, leading to a complete lack or decrease in DNA-binding ability. In the cytoplasm of some cell types, mutant p53 has recently been shown to induce apoptosis through a transcription-independent pathway more effectively than wild-type p53 (45, 46), which is consistent with our results (U87MG vs. T98G cells, hG020 vs. hG008 cells). The decrease of cell proliferation following MIF gene silencing was abrogated by p53 gene knockdown both in glioma cells and BTICs, indicating that the MIF–p53 axis was essential for glioma tumorigenesis based on BTICs. A more detailed analysis of MIF function in BTICs may thus be important, especially when considering the development of drugs targeting MIF.

Histologically immature neoplasms are generally more malignant and aggressive in their development (47). Indeed, BTICs are assumed to be the origin of brain tumors and also to remain in an extremely undifferentiated status. Recently, it has been reported that p53 acts as a barrier to somatic cell reprogramming, a dedifferentiation process resembling tumor formation (48). Our observations suggest the following hypothesis: if p53 in a normal glial cell is directly inactivated by accumulated MIF by factors such as cell stresses, including hypoxia or hypoglycemia, the cell may lose its resistance to dedifferentiation and develop into an immature tumor cell resembling a BTIC, the cell of origin and driving force of tumor growth. In this hypothesis, BTICs may be derived from fully differentiated cells, such as astrocytes, not only from NSCs in the brain. This possibility is supported by at least one recent study (49). Additional evidence also suggests that glioblastoma develops largely in subcortical white matter and rarely in ventricular zone, in which NSCs are abundantly present in adult human brain (50).

In conclusion, we have shown that MIF is highly expressed in BTICs and plays critical roles in the tumorigenicity of BTICs in vitro. Our study further suggests that MIF plays an important role in transforming benign glial cells to BTICs in the niche region, where glial cells are more likely to be exposed to cellular stresses. Moreover, MIF may represent a molecular therapeutic target and clinical marker for glioma therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interests were disclosed.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): R. Fukaya, S. Ohta, T. Yaguchi, E. Sugihara
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