Abstract
Methylation of the O6-methylguanine-DNA methyltransferase (MGMT) gene promoter (i.e., gene silencing) occurs in 40% to 50% of patients with glioblastoma and predicts benefit from temozolomide chemotherapy; when unmethylated, MGMT repairs DNA damage induced by temozolomide, contributing to chemoresistance. In this study, we tested the hypothesis that MGMT is regulated by p53 in astrocytic cells, the precursors of which may give rise to glioblastoma. p53 is of interest because, in addition to often being mutated in glioblastoma, inactivation sensitizes some astrocytoma cell lines to temozolomide. MGMT expression was examined in neonatal murine astrocytes and SF767 human astrocytic glioma cells following p53 inactivation by knockout (murine only) or RNAi methods. MGMT mRNA and protein were detected in murine wild-type p53 astrocytes. However, in knockout murine astrocytes and wild-type cells in which p53 was inhibited by RNAi, MGMT expression was reduced by >90%. This effect of p53 on MGMT expression was unrelated to MGMT promoter methylation—in both wild-type and p53-null astrocytes, the MGMT promoter was unmethylated. In wild-type astrocytes, the p53 protein localized to a regulatory region of the MGMT promoter. In SF767 human astrocytic glioma cells, transient knockout of p53 led to the down-regulation of MGMT gene expression. In murine astrocytes and SF767 cells, p53 regulates MGMT expression without affecting promoter methylation; in astrocytes, this effect may be due to direct binding of p53 to the MGMT promoter. These results imply that the best use of temozolomide requires a thorough understanding of MGMT regulation. [Cancer Res 2007;67(2):580–4]

Introduction
Progress in the treatment of glioblastoma, an aggressive cancer of the brain, has been slow, with only a handful of patients surviving 2 years. A significant insight and therapeutic advance in the management of glioblastoma occurred recently, however, when Stupp et al., observed that the addition of temozolomide chemotherapy to radiotherapy in newly diagnosed cases significantly prolonged tumor control and patient survival (1). Subsequently, by analyzing glioblastoma tissues from study patients, Hegi et al., found that the survival advantage conferred by temozolomide chemotherapy was associated with methylation of the promoter region of the DNA repair gene O6-methylguanine-DNA methyltransferase (MGMT; ref. 2). MGMT repairs DNA damaged by temozolomide, thereby contributing to drug resistance. As such, MGMT methylation status, an indicator of gene silencing, emerges as a potentially important molecular test to determine which patients with glioblastoma will benefit from, and should receive, temozolomide.

From Hegi et al., an important clinical question arises: will a diagnostic test of MGMT promoter methylation be sufficient to identify all patients who will benefit from treatment with temozolomide? To answer this question with certainty, new knowledge will be needed: why is a ubiquitous DNA repair protein like MGMT silenced in some glioblastomas? Which molecules regulate MGMT promoter methylation and expression in glioblastomas? Is methylation of the promoter the primary mechanism of MGMT down-regulation in glioblastomas? In Hegi et al., MGMT promoter methylation was detected in 45% of glioblastomas (2). In this regard, the tumor suppressor p53, a transcriptional regulator mutated in 30% to 50% of glioblastomas (3), may be of interest. Inactivation of p53 has been reported to enhance the sensitivity of some human astrocytoma cell lines to 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) and temozolomide (4), drugs which alkylate O6-guanine, the site of repair by MGMT. Here, we provide evidence that p53 regulates MGMT expression in murine astrocytes, and present data suggesting that p53 may also contribute to the regulation of MGMT gene expression in the human astrocytic glioma cell line, SF767.

Materials and Methods
Cell culture. Cultures of wild-type and knockout p53 astrocytes were prepared from the cerebral cortices of newborn C57bl/6 mice (The Jackson Laboratory, Bar Harbor, ME) with known p53 genotypes using a method adapted from McCarthy and de Vellis (5). Briefly, the cerebral hemispheres were isolated from unanesthetized neonates, dissected free of meninges and blood vessels, and a single cell suspension prepared by enzymatic treatment (0.025% trypsin; Invitrogen, Carlsbad, CA) and mechanical dissociation. Astrocyte cultures were grown in DMEM containing 10% heat-inactivated fetal bovine serum and 50 units/mL penicillin and streptomycin. The status of the p53 gene was assessed by PCR using genomic DNA isolated from mouse tails, following the protocol provided by the supplier. Astrocytes with identical genotypes were pooled from littermates (two to six mice) for each independent experiment. SF767 cells, generously provided by Dr. Mitchell Berger (Brain Tumor Research Center, University of California, San Francisco, San Francisco, CA), were grown in MEM (Sigma, St. Louis, MO) supplemented with 10% FCS.

Fluorescent microscopy. Astrocytes were grown on coverslips for immunostaining. Cells were fixed with 4% paraformaldehyde for 15 min at room temperature and treated with blocking solution (2% goat serum, 0.4% Triton X-100 in PBS) for 10 min. The coverslips were rinsed (0.2% Triton X-100, 0.2% bovine serum albumin in PBS) and exposed to anti-gliial fibrillary acidic protein (GFAP) antibody (Santa Cruz Biotechnology, Santa Cruz, CA), diluted in rinsing solution for 2 h. Subsequently, the coverslips were re-rinsed in PBS, exposed to FITC-conjugated goat anti-mouse
secondary antibody (Santa Cruz Biotechnology) for 1 h, and visualized with a fluorescent microscope (Carl Zeiss Canada, Toronto, ON).

Reverse transcription-PCR. After washing twice with ice-cold PBS, 1 mL of Trizol (Life Technologies, Carlsbad, CA) reagent was added to each culture plate. Samples were removed by scraping and stored at –70°C. RNA extraction was done following the Trizol protocol as described by the manufacturer. PowerScript Reverse Transcriptase (Clontech, Mountain View, CA) was used for reverse transcription as instructed by the manufacturer. PCR was done using Qiangen Hotstart Taq (Qiangen, Valencia, CA) as also instructed. Bands were quantitated using Quantity One software (Bio-Rad, Richmond, CA), readily available on the Internet. The primers used for PCR and MS-PCR were as follows:

Murine p53 forward 5'-CTTACATGTCGAGCATCTGTTG-3', Murine p53 reverse 5'-GTGATTGTGGTATACTCAGAGCC-3', Murine MGMT forward 5'-GGTGTATTAGGAAGCTGCTGA-3', Murine MGMT reverse 5'-GGATTCAGATGTTTTGTCGTA-3', Murine β-2-microglobulin forward 5'-ATGGGAAGCGCAATACCTG-3', Murine β-2-microglobulin reverse 5'-GAAAGACCCAGTCCTTGGTA-3', Murine MGMT unmethylated forward 5'-TTTTGAGTGAAGTTAGTAC-3', Murine MGMT unmethylated reverse 5'-CCCAAACACATACACACAAA-3', Murine MGMT methylated forward 5'-GGTAGTTTTTATAGATTACGTTGCTGCGG-3', Murine MGMT methylated reverse 5'-CAAACGCGTACACGAAATAA-3', Human p53 forward 5'-ACATCTGGCCTTGAAACCAC-3', Human p53 reverse 5'-CGACGACCGCATCCTGAAACGA-3', Human MGMT forward 5'-GTGTTCAGAGCTTGGTGA-3', Human MGMT reverse 5'-ACAGATGTTCCCTTTAGTGCC-3', Human MGMT unmethylated forward 5'-TTTTGGTTTGTATGGTTGTCAC-3', Human MGMT unmethylated reverse 5'-AATCCCAACTCTTCCAAACAAAACCA-3', Human MGMT methylated forward 5'-TCTTTAGCAGTTGTAGGTTTCCG-3', Human MGMT methylated reverse 5'-GACATCCTTCCGAAACGAAAACCA-3', Human β-2-microglobulin forward 5'-GCTATCCAGCGTGTTTTGGTGC-3', Human β-2-microglobulin reverse 5'-GAGTAGTTCCCTTTAGTGCC-3'.

Western blotting. Packed cells were resuspended in an equal volume of radioimmunoprecipitation assay lysis buffer (50 mMol/L Tris, 1% SDS, 150 mmol/L NaCl, 12 mmol/L sodium deoxycholate, 50 mmol/L NaF, 1% Triton X-100, 0.5% NP40, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L DTT, 1 μg/mL aprotinin, and 1 μg/mL pepstatin). The suspension was placed on ice for 15 min, vortexed for 10 s and spun-down for 10 min at 12,000 rpm at 4°C. The supernatant was removed and the protein concentration determined using the Bio-Rad Protein Assay Kit (specification from Bio-Rad). Lysates were supplemented with 500 μmol/L of DTT and protein sample buffer (Invitrogen) and heated to 70°C for 10 min. Samples were loaded and run on precast 4% to 12% gradient SDS-PAGE gels (Bio-Rad, Richmond, CA), readily available on the Internet. The primers used for a sequence of the MGMT gene 5,000 bp downstream of the MGMT promoter. The primers used for the PCR component of the assay were:

- MGMT promoter forward 5'-GCTCACGCCACCTAAAACCTTG-3',
- MGMT promoter reverse 5'-GAGGTTCCTAGAGAAAGAGGA-3',
- Control forward 5'-AGTCCAGTTTCCAGAGAACA-3',
- Control reverse 5'-GCCCTTGAGAAGTAGTGGAGA-3'.

Results

Astrocytes were isolated from the cerebral cortices of 3-day-old wild-type and knockout p53 C57Bl/6 pups and cultured for 96 h as described. Virtually 100% of these brain-derived cells expressed GFAP, affirming their astrocytic nature (Fig. 1). From these astrocyte-enriched cultures, RNA was harvested and RT-PCR done to assess MGMT gene expression. We observed that MGMT gene expression was substantially lower in knockout p53 astrocytes than in wild-type p53 astrocytes (Fig. 2A). To confirm an interaction at the protein level, extracts were prepared from knockout and wild-type p53 astrocytes and the levels of MGMT protein were examined. As seen in Fig. 2B, MGMT protein was detected in extracts from wild-type p53 astrocytes but was not readily discernible from knockout p53 astrocytes, correlating with the RT-PCR data. To ascertain whether the p53-null state was a major contributor to the reduction in MGMT expression, we quantitated the differences in MGMT gene expression in knockout compared with wild-type astrocytes, and noted that an absence of p53 expression was associated with a >90% reduction in MGMT gene expression (data not shown). To confirm that down-regulation of MGMT expression in the absence of p53 was not simply an idiosyncrasy of genetically modified mice, p53 expression was transiently inhibited in wild-type astrocytes by RNAi. We observed that successful inhibition of p53 using a mixture of three different RNAi duplexes specific for p53 (Fig. 3, RNAi) substantially reduced MGMT gene expression. The specificity of this effect on MGMT

1 http://rsat.ulb.ac.be/rsat/.
expression was confirmed using a random duplex (CNTRL), as well as an unrelated RNAi duplex (data not shown). Taken together, these findings further support the conclusion that p53 positively regulates MGMT gene expression in murine astrocytes.

In human glioblastomas, the expression of MGMT seems to be regulated by promoter methylation, an epigenetic modification that prevents transcription factor binding and gene expression (7). Accordingly, we tested the supplementary hypothesis that p53 regulates MGMT expression in murine astrocytes by controlling the methylation status of the MGMT gene promoter. As shown in Fig. 4A, we found no relationship between p53 genotype and MGMT promoter methylation; the MGMT promoter was unmethylated in both wild-type and knockout p53 astrocytes. This finding indicated that p53 regulated MGMT gene expression in murine astrocytes in a manner unrelated to promoter methylation and led us to explore a direct mechanism of interaction. We searched the murine MGMT promoter for the p53 consensus site 5'PuPuPuC(A/T)-(T/A)GPyPyPy-3'. This search yielded a previously unidentified perfect p53 binding site, consisting of two half sites separated by 11 bases in the first intron of the murine MGMT gene (Fig. 4B). Aware that Iwakuma et al., had reported a regulatory region for an E2F-like enhancer element for MGMT expression in this region (8), we used the CHIP assay to explore the possibility that p53 interacted with this DNA segment. As seen in Fig. 4C, the p53 protein was detected at this MGMT regulatory region.

To determine if regulation of MGMT expression by p53 occurred in human cells, we searched the literature for a glioma cell line expressing both functional wild-type p53 and MGMT. We found...
only one human astrocytic glioma cell line, designated SF767, that simultaneously expresses both genes (9–14). Using SF767 cells, we transiently inhibited p53 expression by applying a mixture of two RNAi duplexes. As shown in Fig. 5A, inhibition of p53 by RNAi was accompanied by down-regulation of MGMT gene expression; the average decrease in MGMT expression in three independent experiments was 43% (P = 0.0199). SF767 cultures seemed to contain a mixed population of glioma cells; in some, the MGMT promoter was methylated, whereas in others, the promoter was unmethylated (Fig. 5B). Furthermore, down-regulation of MGMT expression accompanying p53 inhibition by RNAi was not associated with a discernible change in the pattern of MGMT promoter methylation (Fig. 5B). Although additional experiments are required to fully ascertain the functional significance of MGMT down-regulation, early results suggest that p53 inhibition also sensitizes SF767 cells to temozolomide (data not shown). Moreover, we have identified several potential p53 binding sites in the human MGMT gene promoter.

Discussion

Many effective cancer chemotherapeutics alkylate DNA. Although a variety of DNA adducts are generated by such drugs, O6-methylguanine (O6MeG) is a commonly acquired and clinically important lesion. The addition of a methyl group to O6-guanine leads to interstrand cross-linking, faulty DNA replication, and cancer cell death. O6MeG lesions induced by chemotherapeutics can be repaired, however. Repair of O6MeG is mediated by the suicide protein MGMT (15). MGMT restores the structural integrity of DNA by irreversibly transferring methyl adducts to an internal cysteine residue; thereafter, MGMT is degraded by the proteasome. Thus, in the context of cancer treatment, DNA repair by MGMT emerges as a potentially significant determinant of the effectiveness of certain chemotherapies. Indeed, the role of MGMT in resistance to chemotherapy is increasingly apparent. For example, among patients with high-grade astrocytomas (i.e., glioblastoma), the duration of tumor control and patient survival after BCNU or temozolomide are highly associated with evidence of MGMT activity: both MGMT immunonegativity and MGMT promoter methylation predict good tumor control and long survival, whereas active expression of MGMT is associated with early tumor progression and short survival time (2). Yet to be explained, however, is the dichotomous expression of MGMT in histologically indistinguishable glioblastomas. Seeking to clarify this issue, we studied the influence of p53 function on MGMT expression in astrocytes.

We reasoned that p53 might be a regulator of MGMT expression because p53 inactivation has been reported to sensitize astrocytes and astrocytoma cells to BCNU and temozolomide (16, 17) and because p53 mutations and MGMT promoter methylation each occur in ~40% of newly diagnosed glioblastomas. Concerned that glioma cell lines could harbor extraneous genetic abnormalities complicating the analysis, we first studied the association of p53 and MGMT in normal astrocytes cultured from the cerebral hemispheres of neonatal mice. In knockout p53 astrocytes and in wild-type astrocytes in which p53 has been transiently inhibited, MGMT expression is reduced. Quantitative analysis showed a >90% reduction in MGMT expression in knockout versus wild-type cells, suggesting that p53 is a major contributor to MGMT expression in murine astrocytes. These findings are consistent with the earlier observation by Nutt et al. (17) that MGMT activity is significantly decreased and sensitivity to BCNU is significantly increased in p53-null murine astrocytes compared with wild-type p53 astrocytes.

We found no evidence that p53 regulates MGMT expression in murine astrocytes via an effect on MGMT promoter methylation, but discovered a p53 binding site in the murine MGMT promoter. We also observed a direct interaction between the p53 protein and the MGMT promoter. Although there may be promoter-independent mechanisms by which p53 regulates MGMT in murine astrocytes, our data suggest that p53 directly regulates MGMT expression. Indeed, p53 is known to interact with basal transcription machinery (18–20) to regulate the expression of many genes containing a p53 consensus site. Although only one consensus site was found in the murine MGMT promoter, GADD45, a p53 regulated gene, contains a single consensus site (21, 22), as do transforming growth factor-β (23) and IκB-α (24). Hence, a single consensus site is sufficient for p53-mediated transactivation. Furthermore, using less stringent search criteria (i.e., permitting base substitutions in the consensus sequence), we found multiple potential p53 binding sites in the MGMT promoter.

Determining whether p53 also plays a role in regulating the expression of MGMT in human tumors of astrocytic derivation is more challenging because there are few human experimental systems in which to examine the interaction and because correlative studies using human glioblastoma tissues are prone to a degree of uncertainty due to confounding variables. Nevertheless, there are data supporting a role for p53 regulation of MGMT expression in human gliomas. Russel et al., used an alkyltransferase activity assay to monitor the activity of MGMT in human xenografts of anaplastic astrocytomas, glioblastomas, and medulloblastomas (13). They found that the average level of MGMT activity in tumors containing mutant p53 was 198 fmol/mg protein compared with 697 fmol/mg protein in samples containing wild-type p53. Anker et al., found that glioblastomas harboring mutant p53 expressed lower levels of MGMT than tumors containing wild-type p53 (25). Yuan et al., in an immunohistochemical analysis of 35 astrocytomas, noted that expression of mutant p53 correlated with
MGMT immunonegativity; the presence of mutant p53 and MGMT were mutually exclusive (26). Here, we have shown that disruption of p53 in SF767 cells significantly decreases MGMT expression without affecting promoter methylation. Together, these observations support a relationship between p53 and MGMT expression in glioblastomas and encourage further study.

Whether p53 contributes to MGMT regulation in astrocyte precursors or neural stem cells, the likely cells of origin of human glioblastomas, or whether our data are relevant to the treatment of human glioblastomas, where inactivating mutations of p53 and silencing of MGMT are common, remain unanswered. Nevertheless, the demonstration that p53 modulates MGMT expression in murine astrocytes and the human astrocytic glioma cell line, SF767, raises the possibility that p53 function, or the p53 pathway, may be another determinant of MGMT activity. This observation potentially sheds light on long-surviving glioblastoma cases that tested negative for MGMT promoter methylation in the analysis done by Hegi et al. (2). Clearly, knowing all the mechanisms of MGMT regulation in gliomas will be critical to making the very best chemotherapy treatment decisions for patients with glioblastoma.

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O\textsuperscript{6}-Methylguanine-DNA Methyltransferase Regulation by p53 in Astrocytic Cells

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