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Epigenetic Regulation of the miR142-3p/Interleukin-6 Circuit in Glioblastoma

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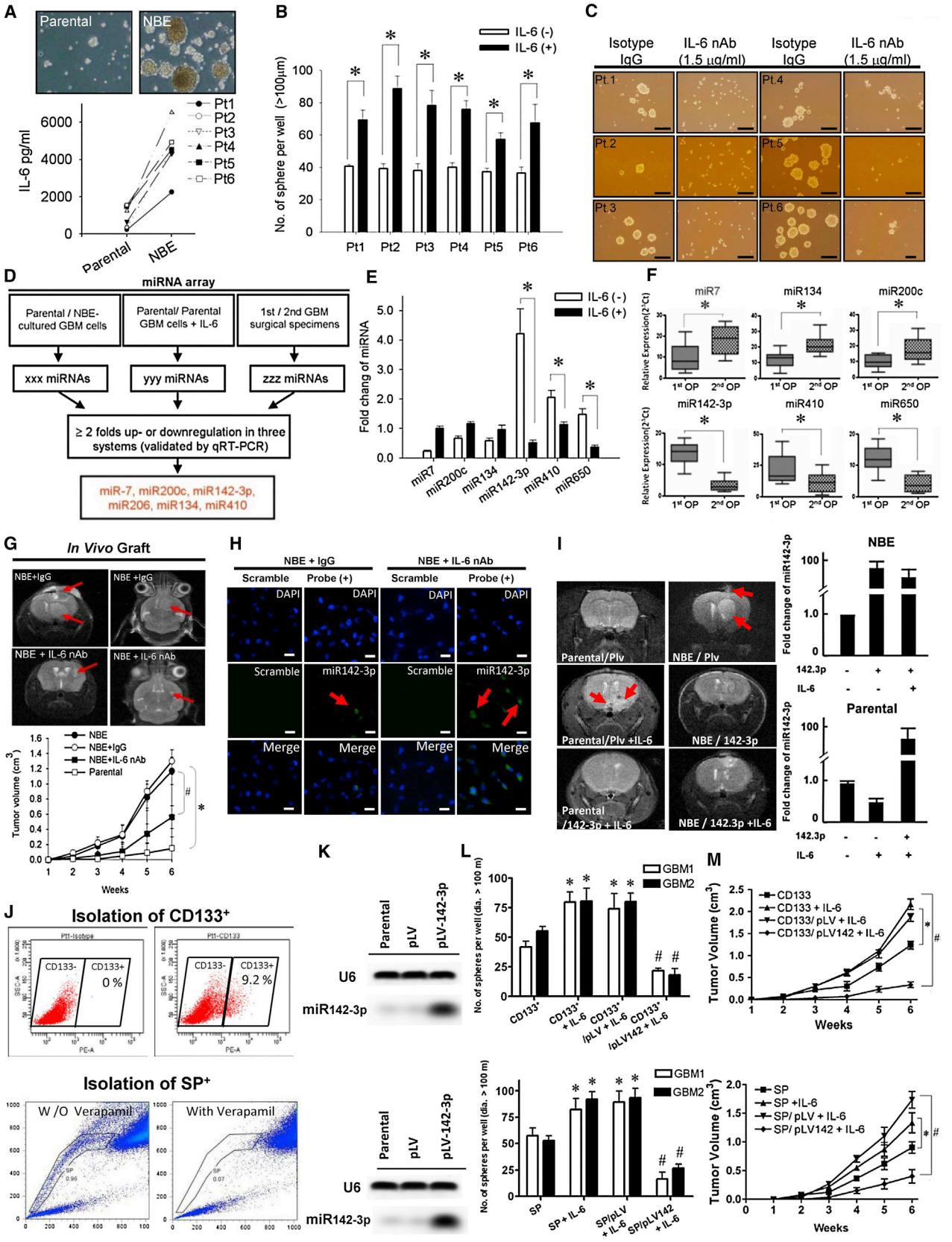
SUMMARY

Epigenetic regulation plays a critical role in glioblastoma (GBM) tumorigenesis. However, how microRNAs (miRNAs) and cytokines cooperate to regulate GBM tumor progression is still unclear. Here, we show that interleukin-6 (IL-6) inhibits miR142-3p expression and promotes GBM propagation by inducing DNA methyltransferase 1-mediated hypermethylation of the miR142-3p promoter. Interestingly, miR142-3p also suppresses IL-6 secretion by targeting the 3′ UTR of IL-6. In addition, miR142-3p also targets the 3′ UTR and suppresses the expression of high-mobility group AT-hook 2 (HMGA2), leading to inhibition of Sox2-related stemness. We further show that HMGA2 enhances Sox2 expression by directly binding to the Sox2 promoter. Clinically, GBM patients whose tumors present upregulated IL-6, HMGA2, and Sox2 protein expressions and hypermethylated miR142-3p promoter also demonstrate poor survival outcome. Orthotopic delivery of miR142-3p blocks IL-6/HMGA2/Sox2 expression and suppresses stem-like properties in GBM-xenotransplanted mice. Collectively, we discovered an IL-6/miR142-3p feedback-loop-dependent regulation of GBM malignancy that could be a potential therapeutic target.

INTRODUCTION

Glioblastoma multiforme (GBM) is the most aggressive primary brain tumor with a poor prognosis (Stupp et al., 2005). Due to its malignant progression and widespread invasion throughout the brain, GBM is highly resistant to traditional chemoradiotherapies and newly developed targeted therapies (Stupp et al., 2007). A number of microRNAs (miRNAs) have been identified that target oncogenes, cell cycle regulators, and transcription factors involved in brain tumors (Gillies and Lorimer, 2007). For example, miR7, miR21, miR26a, miR124, miR137, miR184, and miRNA221/222 are implicated in GBM pathogenesis; others, such as miR10b and miR26a, appear to be prognostic markers of high-grade glioma (Hermansen and Kristensen, 2013; Møller et al., 2013). Interestingly, miR34a inhibits cell cycle progression, migration, and survival and induces differentiation of glioma stem cells, but not normal brain astrocytes (Møller et al., 2013), suggesting that some miRNAs may be particularly important for cancer stem-like cells. Nonetheless, the underlying mechanisms of miRNAs in GBM progression and stem-like properties still remain unclear.

Inflammatory cytokines in the tumor microenvironment are critical mediators regulating tumor growth, metastasis, and



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drug resistance (Korkaya et al., 2012; Straussman et al., 2012). Interleukin-6 (IL-6) has been linked to tumorigenesis due to its positive effect on tumor growth and angiogenesis and its negative effect on patient survival rate (Iliopoulos et al., 2009; Korkaya et al., 2012; Sansone et al., 2007). The role of IL-6 in the malignancy of glioma was demonstrated in IL-6-deficient and Src-overexpressing transgenic mice (Weissenberger et al., 2004). A small population of brain tumor cells with mutated epidermal growth factor receptor (EGFR) was shown to induce IL-6 biosynthesis and secretion into the tumor microenvironment, resulting in accelerated growth of the entire tumor mass (Inda et al., 2010). In breast cancer cells, IL-6 was shown to drive transformation by suppressing miR200c in a paracrine manner (Rokavec et al., 2012). In cholangiocarcinomas, IL-6 contributes to tumor growth by epigenetically modulating miR370 (Meng et al., 2008) and to oncogenesis by increasing the activity of DNA methyltransferase 1 (DNMT1) (Braconi et al., 2010). The underlying mechanism of epigenetic regulation mediated by IL-6 or other cytokines in the GBM malignancy is not well understood.

miR142 was first reported to regulate hematopoiesis and T cell development (Chen et al., 2004). Studies later demonstrated that miR142-3p directly targets the 3′ UTR of IL-6 in dendritic cells (Sun et al., 2011) and that IL-6 expression decreases in hematopoietic cells in a miR142-3p-dependent manner (Sun et al., 2013). During cardiac growth in response to hemodynamic stress, downregulation of miR142 is required to enable cytokine-mediated survival signaling (Sharma et al., 2012). In acute myeloid leukemia (AML) miR142-3p is downregulated; thus, increasing the expression of miR142-3p has been suggested as a potential strategy for AML treatment by promoting myeloid differentiation (Wang et al., 2012). Similarly, miR142-3p has also been shown to inhibit tumor progression and invasion in hepatoma (Wu et al., 2011). However, the role of miR142-3p or the miR142-3p/IL-6 circuit in GBM is still undetermined. Here, we further examine the role of the miR142-3p/IL-6 circuit and its potential contribution to GBM tumorigenesis. Our findings reveal a miR142-3p-driven autocrine and paracrine positive loop that epigenetically regulates the progression and cancer stem-like property of GBM.

RESULTS

IL-6 Suppresses miR142-3p Expression and Enhances GBM Aggressiveness

Inflammatory cytokines in the tumor microenvironment play a key role in regulating tumor malignancy. The highly malignant and dismal prognosis of GBM is partially attributed to its high degree of heterogeneity and the persistence of a small population of cancer stem-like cells (CSCs). However, the underlying mechanisms of cytokine-regulated GBM progression or GBM-related CSCs remain unclear. A previous study reported an optimal "NBE" condition (serum-free neurobasal media supplemented with fibroblast growth factor [FGF]/epidermal growth factor [EGF]) that allows GBM cells to express several characteristics of neural stem cell phenotypes and genotypes (Lee et al., 2006). We isolated GBM cells from 6 patients (Pt 1–6) and cultured them in serum-containing medium (Dulbecco's modified Eagle's medium [DMEM] + 10% fetal bovine serum [FBS]) or NBE for ten passages to establish parental and NBE-GBM cells. These NBE-GBM cells exhibited a remarkable ability for tumorsphere formation, which represents a self-renewal capacity, compared with parental cells (Figure 1A, top). To investigate whether cytokines may be secreted in a paracrine and autocrine manner to mediate the stem-like phenotypes in GBM cells, a cytokine antibody array was performed, which revealed that NBE-cultured GBM cells secreted higher amounts of IL-6 into the culture medium than did the parental cells (Figures 1A and S1A available online). Transferring parental GBM cells to NBE medium supplemented with recombinant IL-6 restored the sphere-forming ability of parental GBM (Figure 1B). Notably, adding IL-6-neutralizing antibodies (IL-6-nAbs) to NBE medium consistently abrogated the sphere-forming ability of NBE-GBM (Figures 1C and S1C). These results suggest that

Figure 1. Secreted IL-6 and IL-6-Suppressed miR142-3p Play Critical Roles in GBM Aggressiveness

(A) Top: GBM cells from six patients (Pt1–Pt6) were isolated and grown in serum-containing or NBE medium for 10 passages to establish the parental and NBE-GBM cells (bar, 200 μm). Bottom: IL-6 protein levels were detected by ELISA in the conditioned medium derived from parental and NBE-GBM cells.

(B) Quantification of the number of spheres from NBE-GBM cultured with or without 1.5 μg/ml of IL-6 for 1 week. Error bars indicate mean ± SD from three independent experiments. *$p < 0.01$.

(C) Representative images of sphere formation of NBE-GBM treated with 1.5 μg/ml of immunoglobulin G (IgG) antibody control or IL-6-nAB.

(D) The process of identifying putative miRNAs associated with GBM aggressiveness and IL-6 regulation.

(E) miRNAs identified from isolated GBM cells were confirmed by qRT-PCR. Error bars indicate mean ± SD from three independent experiments. *$p < 0.01$.

(F) qRT-PCR of miRNA expression profiles from the first and second OP samples of GBM patients. *$p < 0.01$.

(G) Immunocompromised mice were orthotopically transplanted with parental GBM, NBE-GBM, IgG-treated NBE-GBM, or IL-6-nAB-treated NBE-GBM cells. Tumor size was monitored by MRI brain scans for 6 weeks. Error bars indicate mean ± SEM; $n = 6$. *$p < 0.01$; #$p < 0.05$.

(H) FISH staining of miR142-3p in NBE-GBM cells treated with IgG control or IL-6-nAB. Bar, 200 μm.

(I) Immunocompromised mice were orthotopically transplanted with parental GBM or NBE-GBM cells, followed by miR142-3p delivery and/or IL-6 treatment (1.5 μg/ml) as indicated ($n = 6$). Representation of MRI whole-brain scans (left). MiR142-3p delivery efficiency was validated by qRT-PCR (right). Error bars indicate mean ± SD from three independent experiments.

(J) Isolation of CD133-positive subset ($CD133^+$; top) or side population (SP^+; bottom) by flow cytometry from isolated GBM patient specimens.

(K) Northern blot of miR142-3p expression in GBM-$CD133^+$ or GBM-SP^+ overexpressing miR142-3p pLV-lentiviral transfection.

(L) Effect of IL-6 on the sphere-forming ability of GBM-$CD133^+$ (top) or GBM-SP^+ (bottom) with or without miR142-3p transfection. Error bars indicate mean ± SD from three independent experiments. *$p < 0.01$; #$p < 0.01$.

(M) Effect of IL-6 on in vivo tumor growth of GBM-$CD133^+$ (top) or GBM-SP^+ (bottom) with or without miR142-3p transfection. Error bars indicate mean ± SEM; $n = 6$. *$p < 0.01$; #$p < 0.01$.

See also Figures S1 and S2.

IL-6 plays a critical role in regulating GBM progression or GBM-related CSC.

IL-6 has been shown to suppress miR200c, leading to an activation of the inflammatory signaling circuit and tumorigenesis (Rokavec et al., 2012). We further elucidated the miRNAs involved in IL-6-mediated GBM malignancy by subjecting three sets of GBM paired groups: parental GBM versus NBE-GBM, parental cells in NBE medium versus parental cells in NBE medium plus IL-6, and GBM specimens from patients receiving the first versus second operation to complementary miRNA microarray analyses (Figure 1D). The candidate miRNAs were either upregulated or downregulated by $\geq$2-fold (Figure 1D). Among those downregulated, miR142-3p was the most significant and correlated with IL-6 treatment (Figure 1E) and second operation of GBM specimens (Figures 1F and S1E). Quantitative RT-PCR (qRT-PCR) analysis of the six NBE-cultured cell lines showed that IL-6 neutralization (IL-6-nAb) substantially elevated miR142-3p expression (Figures S1F and S1G), while IL-6 treatment suppressed cellular miR142-3p expression (Figure S1G). The IL-6-nAb treatment also decreased sphere and tumorigenic colony formation in these six NBE-cultured cell lines (Figures S2A and S2B). MRI of an in vivo tumorigenesis assay revealed severe tumor growth, which can be blocked by preculturing with IL-6-nAb, in recipients of NBE-cultured GBM cells 6 weeks after xenotransplantation (Figure 1G). Fluorescence in situ hybridization (FISH) assay demonstrated that IL-6-nAb restored the expression of miR142-3p in NBE-cultured GBM cells (Figure 1H). Consistent with these results, mice that received miR142-3p-overexpressing NBE-cultured GBM cells showed a substantially suppressed NBE-mediated effect, and presupplementation of IL-6 only mildly restored the NBE-mediated effect in the presence of miR142-3p overexpression (Figures 1I, and S2C, and S2D). These findings suggest that miR142-3p suppresses the tumorigenic potential of GBM.

To further determine whether the miR142-3p/IL-6 circuit occurs in the cancer stem-like subpopulation of GBM cells, we isolated CSC-like GBM cells from GBM patient specimens according to the CD133 surface marker expression ($CD133^+$) and side population (SP^+) identification (Figure 1J). Similar to NBE-GBM cells, isolated GBM-$CD133^+$ (Figure S2E) and GBM-SP^+ (Figure S2F) all exhibited low levels of miR142-3p. Functional analysis showed that overexpression of miR142-3p (Figure 1K) inhibited the sphere-forming ability (Figure S2G) and reduced the percentage of GBM-$CD133^+$ and GBM-SP^+ (Figures S2H and S2I). In contrast, IL-6 treatment increased the sphere-forming ability of both GBM-$CD133^+$ and GBM-SP^+, while overexpression of miR142-3p abrogated these IL-6-induced effects (Figure 1L). In parallel, the increased xenograft tumor-propagating ability of GBM-$CD133^+$ and GBM-SP^+ by IL-6 pretreatment was abrogated by miR142-3p delivery (Figure 1M). Together, these results suggest that IL-6 inhibits miR142-3p, a suppressor of GBM tumorigenesis and GBM stem-like cells, leading to GBM malignancy.

IL-6 Regulates Methylation of miR142-3p Promoter in GBM Progression

IL-6 overexpression has been linked to promoter methylation of miRNAs and contributes to tumor growth by methylation-dependent regulation of miRNAs in cancers (Meng et al., 2008), but the causative relationships between IL-6 and miRNAs remain to be established. Thus, we hypothesized that the IL-6-dependent modulation of miR142-3p expression involves DNA methylation in putative regions of miR142-3p promoter. We first searched for CpG islands in the promoter region of miR142-3p and found 17 CpG dinucleotides (+150 to +500 bp) predicted to be methylated by CpG plot (Figure S3A). Bisulfite sequencing showed that most of these CpG dinucleotides were hypermethylated in 6 pairs of parental and NBE-GBM cells (Figure 2A). Specificity protein 1 (Sp1), a transcription factor upregulated in glioma, has been shown to promote cell invasion by enhancing IL-6-mediated transactivation of vascular endothelial growth factor (VEGF) through direct binding on the promoter in GBM cells (Guan et al., 2012). Remarkably, we found that methylation (black circles) in the promoter regions, especially region 3, which includes a putative Sp1-binding sequence (+181 to +190 bp; Figure S3A), was relatively higher in NBE-GBM cells than in parental GBM cells and was enhanced upon IL-6 treatment in both parental and NBE-GBM cells (Figure 2A). To determine whether methylation of the CpG dinucleotides within the Sp1-binding site is critical for miR142-3p expression, we conducted in vitro methylation assays using luciferase reporters containing full-length or deleted forms of the miR142-3p minimal promoter (−150 to +400 bp). These promoters were methylated in vitro by CpG methyltransferase M. SssI (specifically methylates CpG dinucleotides) or DNA adenine methyltransferase (Dam MT; specifically methylates ApT dinucleotides; a negative control) (Deng et al., 2004) before transfection in NBE-GBM cells for luciferase reporter assay. The results showed that miR142-3p promoter activities were reduced upon premethylation by M. SssI, but not Dam MT (Figure 2B), while premethylation by M. SssI did not inhibit miR142-3p promoter activities when the CpG dinucleotides within the Sp1-binding site were deleted (Figure 2B). To further elucidate the role of the Sp1-binding site on miR142-3p promoter activity, we constructed a luciferase reporter containing mutated miR142-3p promoter (+181 to +190; Figure 2C). Our data showed that mutated Sp1-binding sequences in the reporter vector abolished Sp1-dependent activation, suggesting that Sp1-binding site is essential for the miR142-3p promoter activity. Taken together, these data suggest that Sp1 and the SP1-binding site play critical roles in regulating the promoter activity of miR142, and the binding affinity of SP1 is further suppressed by methylation of CpG dinucleotide in the Sp1-binding site, resulting in the suppression of miR142 promoter activity.

IL-6 Regulates the SP1-Binding Site of the miR142-3p Promoter in a DNMT1-Dependent Manner

IL-6 has been shown to modulate DNMT1 activity and subsequently regulate the activity of miRNAs, such as miR148 and miR152 (Braconi et al., 2010). DNMT1 interacts directly with the transcription factor p53 and is involved in p53-mediated gene regulation (Estève et al., 2005). Moreover, DNMT1 and p53 synergistically repress Sp1-mediated survivin gene expression (Estève et al., 2007). To determine whether the DNMT family is also involved in IL-6-regulated methylation of the miR142-3p promoter, we treated primary GBM cells with IL-6 and found

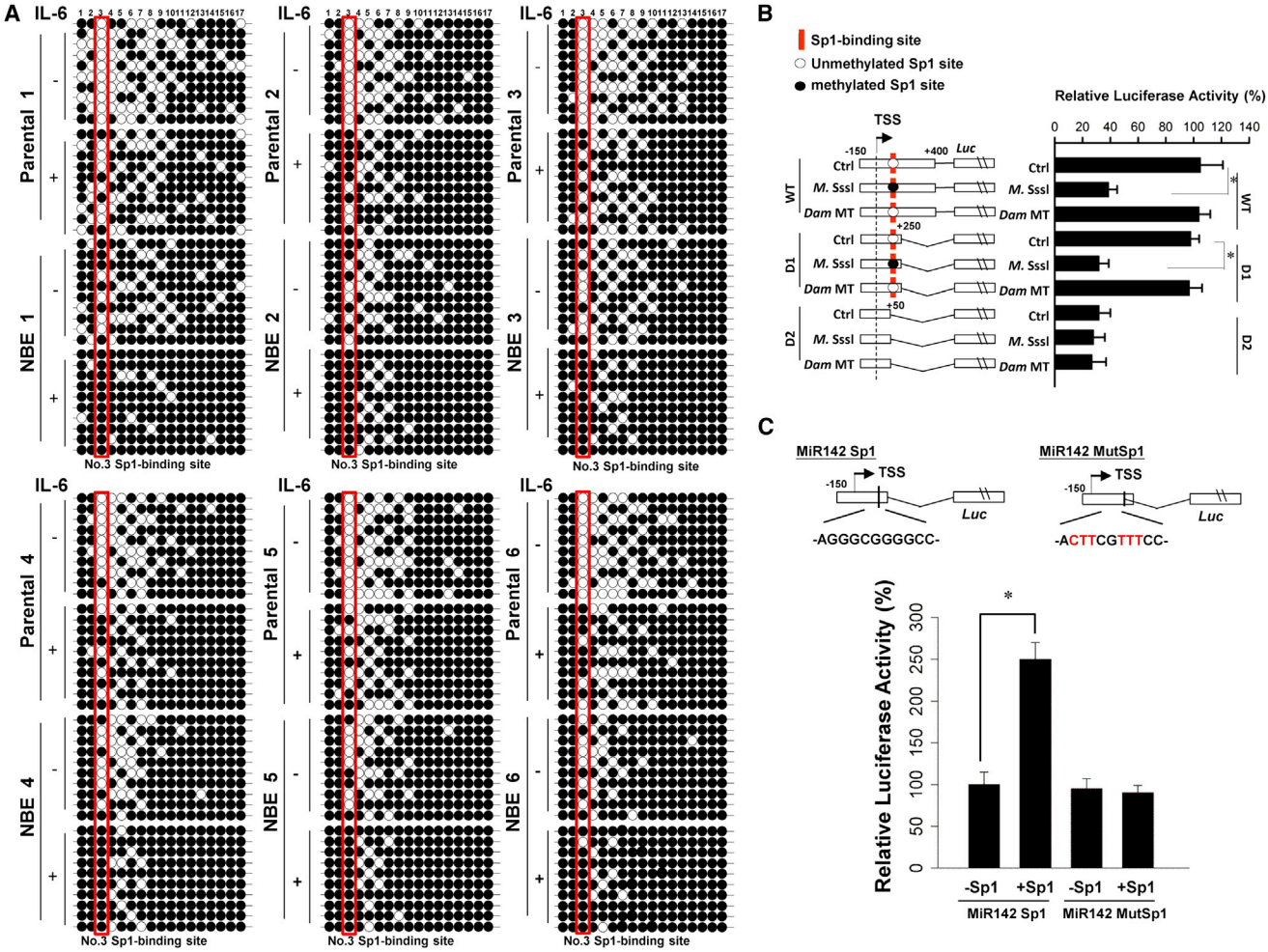

Figure 2. IL-6 Promotes Hypermethylation of the Sp1-Binding Site in the miR142-3p Promoter

(A) Methylation of the CpG dinucleotides of DNA sequences (+150 to +500 bp) downstream of the miR142 transcription start site was detected by bisulfate sequencing. Open circles (○), nonmethylated cytosine; closed circles (●), methylated cytosine (No. 1–17). Six paired NBE cell lines and parental cell lines derived from the same six GBM patients were compared side by side with or without IL-6 (red box, Sp1-binding site).

(B) Full-length and serial deletion constructs of the miR142 promoter in vitro methylated with control (ctrl), M. SssI, or Dam MT (left). Luciferase reporter activity of in vitro methylated expression vectors normalized to expression of *Renilla* firefly luciferase.

(C) Schematic (top) and luciferase reporter activities (bottom) of miR142 Sp1 and miR142 MutSp1 expression vectors.

Error bars in (B) and (C) indicate mean ± SD from three independent experiments. *p < 0.01. See also Figure S3.

that it increased DNMT1, but not DNMT3a or DNMT3b, protein levels (Figure 3A). Knockdown of DNMT1 (shDNMT1), but not DNMT3a (shDNMT3a) or DNMT3b (shDNMT3b), significantly increased the level of miR142-3p in NBE-GBM regardless of IL-6 treatment (Figure 3B). Methylation of the Sp1 site (+181 to +190 bp; CpG dinucleotides) in NBE-GBM cells was consistently suppressed by shDNMT1 (Figures 3C and S3B). Several reports have previously described a DNMT/SP1 protein interaction that recruits DNMT1 to the Sp1 site and results in epigenetic regulation of target genes in glioma cells (Bostick et al., 2007; Hervouet et al., 2010; Sharif et al., 2007). We performed a quantitative chromatin immunoprecipitation (qChIP) assay and found that DNMT1 bound to the SP1 site in the miR142-3p promoter in NBE-GBM cells, and this interaction was increased by IL-6 treatment but suppressed by IL-6-nAb (Figure 3D).

DNMT1 and Sp1 are corecruited to and regulate the methylation of the SLIT2 and HOXA2 promoters (Hervouet et al., 2010). We further investigated whether Sp1 is essential for miR142-3p transcription activation and found that knockdown of Sp1 (shSp1) suppressed miR142-3p level, whereas knockdown of DNMT1 increased it, in NBE-GBM regardless of IL-6 treatment (Figure 3E). Notably, shDNMT1 was no longer able to increase miR142-3p expression in NBE-GBM when cotransfected with shSp1 (Figure 3E). Results from qChIP assay also demonstrated a direct binding of Sp1 to the Sp1 site in the miR142-3p promoter, and this binding was reduced by IL-6 treatment in NBE-GBM cells (Figure 3F). Re-ChIP experiments revealed that both DNMT1 and Sp1 interacted at the Sp1-binding site in the miR142-3p promoter, and IL-6 treatment suppressed Sp1 binding to the Sp1-binding site

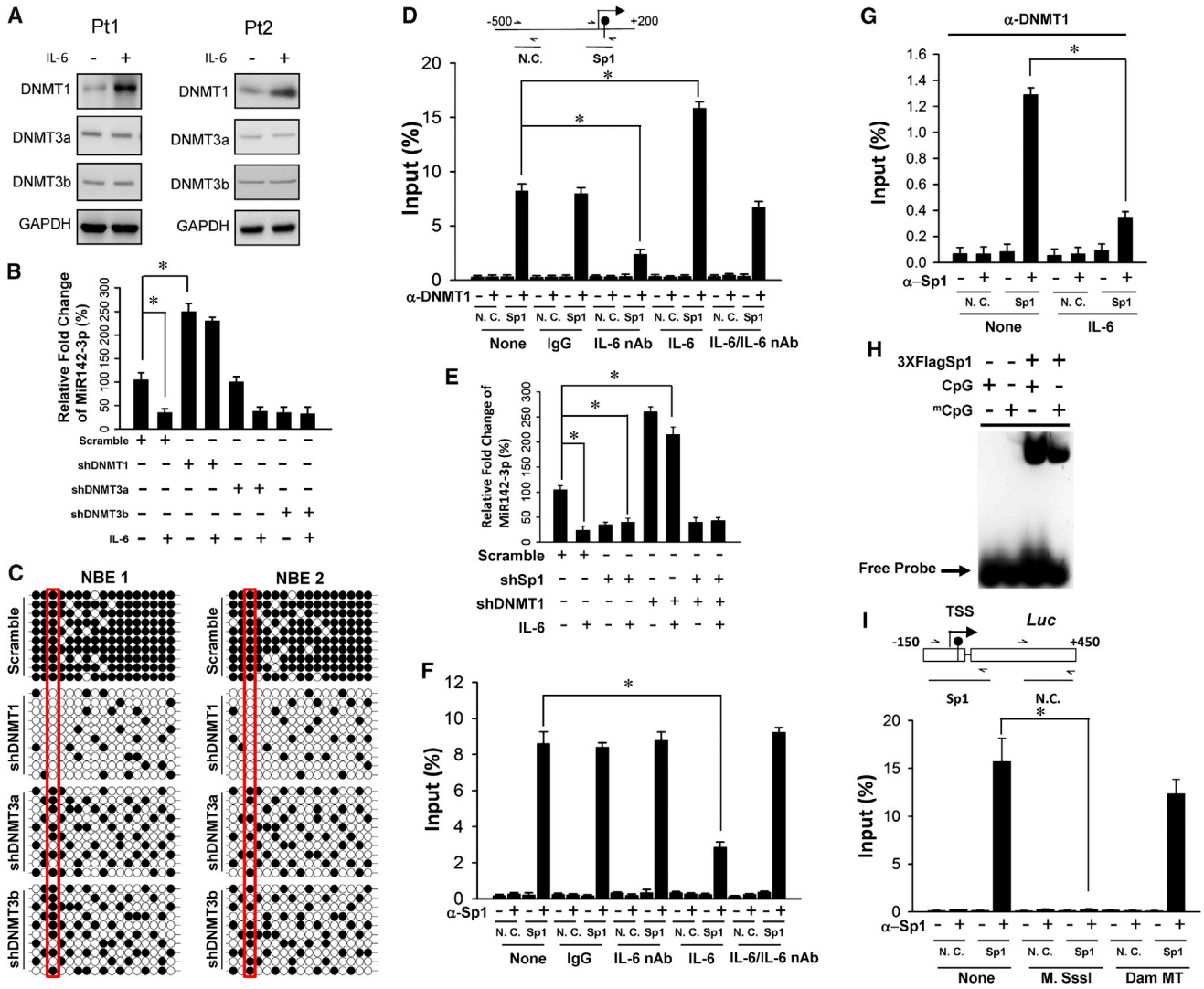

Figure 3. IL-6 Reduces miR142-3p Expression by DNMT1-Mediated Methylation of an Sp1-Binding Site in the miR142-3p Promoter

(A) Western blotting of DNMT1, DNMT3a, and DNMT3b in two representative GBM cells treated with or without IL-6.

(B) TaqMan-based qPCR of miR142-3p levels in cells transfected with scrambled shRNA, shDNMT1, shDNMT3a, or shDNMT3b.

(C) Methylation status of the CpG dinucleotides of DNA sequences (+150 to +500 bp) downstream of the miR142 transcription start site was validated by bisulfate sequencing. ○, nonmethylated cytosine; ●, methylated cytosine; red box, Sp1-binding site.

(D) qChIP-PCR of NBE cells pretreated with control, IL-6, IL-6-nAb, and IL-6+IL-6-nAb using anti-DNMT1. N.C., nonspecific region control; Sp1, Sp1-binding region.

(E) qRT-PCR analysis of miR142-3p levels in NBE-GBM cells were transfected with shSp1 and/or shDNMT1 and treated with or without IL-6.

(F) qChIP-PCR of NBE-GBM cells pretreated with control, IL-6, IL-6-nAb, and IL-6+IL-6-nAb by using anti-Sp1. IgG was used as a control.

(G) NBE-GBM cells pretreated with or without IL-6 were subjected to ChIP by anti-DNMT1 followed by re-ChIP with anti-Sp1. The DNA fragments were analyzed by qPCR.

(H) EMSA was performed using Sp1 and methylated or unmethylated DNA probes containing the +181 to +190 bp region of the miR142-3p gene sequence.

(I) miR142 promoters containing the Sp1-binding site were premethylated in vitro by M. SssI or Dam-MT before cotransfection with Sp1 expression vector in NBE-GBM cells. A qChIP assay was performed with anti-Sp1 and qPCR primers specific for the Sp1 region or nonspecific region control.

Error bars in (B), (D)–(G), and (I) indicate mean ± SD from three independent experiments. *$p < 0.01$. See also Figure S3.

(Figure 3G). An in vitro binding assay using methylated and unmethylated probes containing the Sp1-binding CpG dinucleotides showed that Sp1 bound to the unmethylated probes (Figure 3H). Similarly, premethylation at the Sp1 site by M. SssI inhibited Sp1 binding (Figure 3I). Taken together, these results indicate that IL-6 induces methylation of the miR142-3p promoter to decrease the binding of Sp1 at the Sp1-binding site and decrease miR142-3p expression in a DNMT1-dependent manner.

miR142-3p Regulates the IL-6-Stat3 Loop by Targeting the 3′ UTR of IL-6

Since miR142-3p expression and GBM aggressiveness are negatively correlated, we were prompted to examine the effect

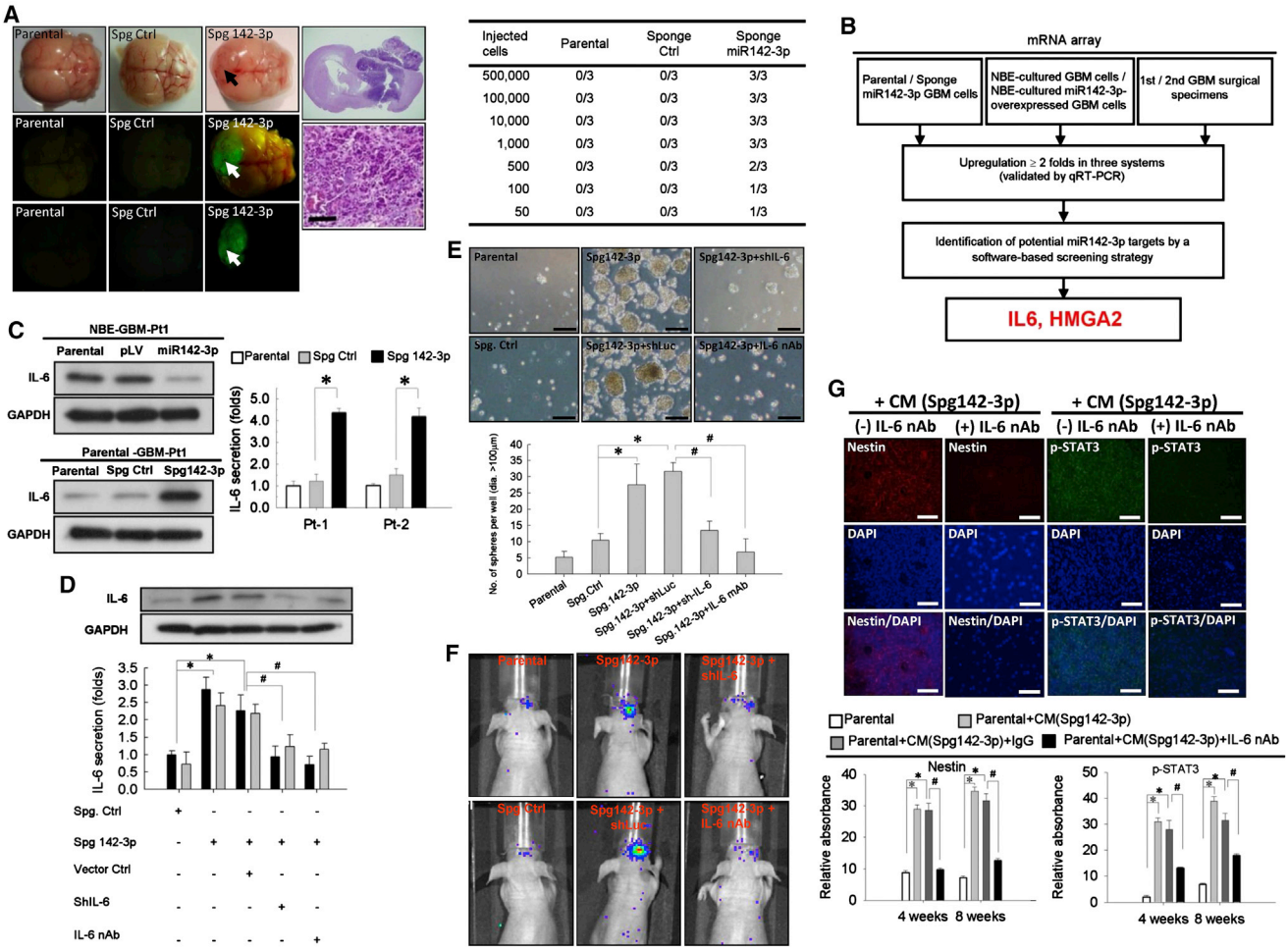

Figure 4. miR142-3p Regulates an IL-6-Dependent Paracrine Loop to Promote GBM Tumorigenesis

(A) Different doses of parental GBM (pGBM), pGBM/Spg-Ctrl, and pGBM/Spg-miR142-3p cells were implanted into mice. The brain images (arrows, tumor lesions; GFP signal represents tumor site) and histological section were acquired 12 weeks after transplantation. Bar, 200 μm.

(B) Identification of putative genes associated with miR142-3p expression.

(C) pGBMs were transfected with the sponge control (Spg-ctrl) or miR142-specific sponge (Spg142-3p). NBE-GBMs were transfected with pLV empty vector (pLV) or miR142-3p plasmid and subjected to western blot (left) and ELISA (right) to assess IL-6 expression and secretion levels, respectively.

(D) IL-6 expression (western blot; top) and secretion (ELISA; bottom) levels in pGBMs exposed to CM derived from pGBMs transfected with Spg-miR142 and shIL-6 and treated with or without IL-6-nAB. Black bars, patient 1; gray bars, patient 2.

(E) Quantification of sphere formation in pGBMs exposed to CM from pGBMs transfected with Spg-miR142-3p and shIL-6 and treated with or without IL-6-nAB.

(F) Tumorigenesis of mice bearing xenotransplantation of pGBM exposed to CM from pGBM transfected with Spg-miR142-3p and shIL-6 and treated with or without IL-6-nAB.

(G) Immunofluorescent staining (top) and quantitation (bottom) of Nestin and p-STAT3 in xenografted tumors from recipients of Spg-miR142-3p-transfected pGBM exposed to CM with or without IL-6-nAB. Bars, 200 μm.

Error bars in (C), (E), and (G) indicate mean ± SD from three independent experiments. *$p < 0.01$; #$p < 0.01$. See also Figures S4 and S5.

of miR142-3p in tumor formation. Using a sponge strategy (Yang et al., 2012), endogenous miR142-3p in parental GBM cells was knocked down (Spg-miR142-3p). Spg-miR142-3p increased the number of soft agar colonies and spheres (Figure S4A) and the percentage of side population cells (Figure S4B) compared with cells transfected with scrambled sponge control and parental cells. Furthermore, compared with scrambled sponge control, we found that silencing endogenous miR142-3p increased the tumor-forming ability of parental GBM cells in xenotransplanted, immunocompromised mice (Figures 4A and S4C). Notably, Spg-miR142-3p increased the tumor-forming ability of parental GBM cells in immunocompromised mice with as few as 50 Spg-miR142-3p-transfected GBM cells, but this was not seen in mice injected with parental or Spg-Ctrl-transfected cells, even with as many as 500,000 cells (Figure 4A). To determine the mechanism of this miR142-3p-dependent tumor propagation, we searched for downstream targets of miR142-3p by mRNA array analysis and a software screening strategy (Figures 4B and S4D). Among all candidate targets, IL-6 and high-mobility group AT-hook 2 (HMGA2) were identified

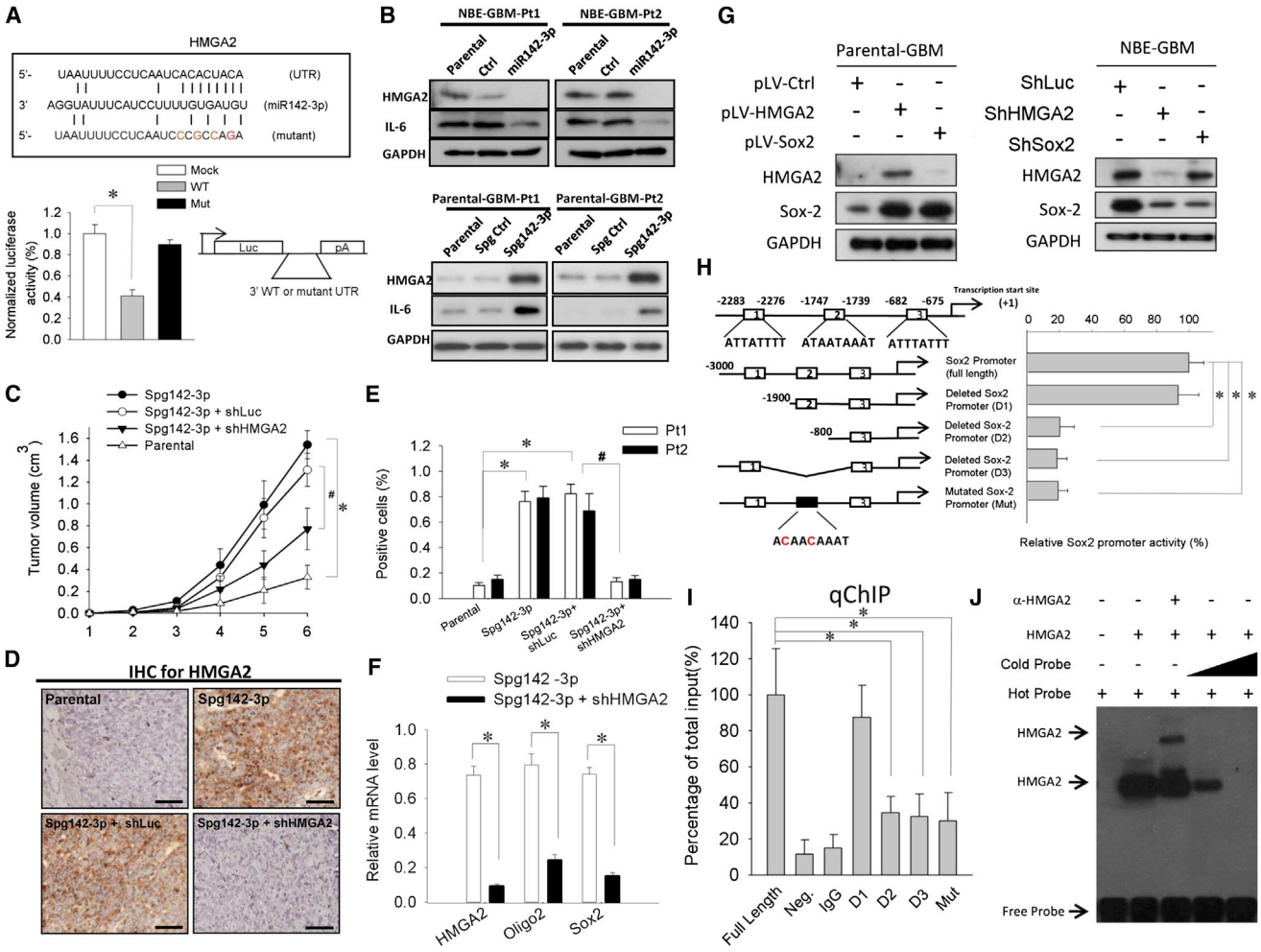

Figure 5. HMGA2 Is a Dominant Effector of the miR142-3p/HMGA2/Sox2 Axis and Binds Directly to the Sox2 Promoter

(A) Top: the putative miR143-binding site in the HMGA2 3′ UTR and the mutation of four specific residues in the seed region of the miR142-3p target site. Bottom: luciferase reporter assay of wild-type and mutated HMGA2 3′ UTR GBM cells were cotransfected with luciferase and β-galactosidase reporter plasmids.

(B) Western blots of HMGA2 and IL-6 in two NBE-GBMs transfected with miR142-3p or pLV vector control (top) and in two pGBMs transfected with Spg-miR142-3p or Spg-ctrl (bottom).

(C) Tumor volume of GBM xenograft in mice that received pGBMs or Spg-miR142-3p-GBMs treated with shHMGA2 or shLuc. Error bars indicate mean ± SEM; n = 6. *p < 0.01; #p < 0.01.

(D–F) Shown are representative images (D) and quantification (E) of IHC for HMGA2 expression and qRT-PCR for HMGA2 and Sox2 (F) in GBM tumor xenografts from recipients of Spg-miR142-3p-GBMs transfected with shLuc or shHMGA2. Bars, 200 μm.

(G) Western blot of HMGA2 and Sox2 of a pGBM clone from patient 1 transfected with HMGA2 and Sox2 (left) or a NBE-GBM clone from the same patient with high endogenous HMGA2/Sox2 expression was transfected with shHMGA2 and shSox2 (right).

(H) Sox2 promoter reporter constructs (left). Full-length (containing three AT-hook regions), deleted, and mutated Sox2 promoter reporter constructs were subjected to luciferase reporter assay (right).

(I) qChIP-PCR of full-length, deleted, and mutated Sox2 promoter regions in the presence of IgG control antibody or anti-HMGA2. Input, 2% of total lysate.

(J) EMSA and competition assay were performed with the nuclear extracts from GBMs transfected with the vector controls with a α^{32}P-dCTP-labeled probe detecting the Sox2 promoter region containing an HMGA2 binding site or unlabeled probe and anti-HMGA2. No protein extract was added to lane 1. Unlabeled probe was added at concentrations 10-fold (lane 4) or 50-fold (lane 5) greater than the labeled probe.

Error bars in (A), (E), (F), (H), and (I) indicate mean ± SD from three independent experiments. *p < 0.01; #p < 0.01.

as potential mediators (Figures 4B, S4D, and S4E). Knockdown of HMGA2 and/or IL-6 suppressed the Spg-miR142-3p-increased tumor propagation of GBM cells in immunocompromised mice (Figure S4C).

To determine if miR142-3p targets IL-6 at its 3′ UTR in NBE-GBM cells, we mutated the 3′ UTR of IL-6 and found that the mutant hampered the inhibitory effect of miR142-3p (Figure S5A). Western blot analysis and ELISA assay showed that the expression and secretion of IL-6 in parental GBM cells were increased by Spg-miR142-3p but decreased by overexpression of miR142-3p in NBE-GBM cells (Figures 4C and 5B). To investigate how the IL-6/miR142-3p regulatory loop contributes to

tumorigenicity, we incubated GBM cells in conditioned medium (CM) derived from a panel of parental GBM cells transfected with Spg-miR142-3p and/or IL-6-shRNA (shIL-6) and with or without IL-6-nAb. CM from Spg-miR142-3p-transfected parental GBM cells increased IL-6 expression and secretion (Figure 4D) and sphere-forming ability (Figure 4E), which were largely suppressed by cotransfecting shIL-6 in CM-providing cells or by adding IL-6-nAb into the CM (Figures 4D and 4E). Preincubating parental GBM cells with CM derived from Spg-miR142-3p-transfected cells substantially increased their tumorigenicity (Figures 4F and S5C) and deteriorated survival (Figure S5D) of the recipients of these cells 6 weeks after xenotransplantation. In contrast, the suppression by shIL-6 or addition of IL-6-nAb to the CM reversed these effects (Figures 4F, S5C, and S5D). Immunofluorescence staining also showed that xenografted tumors of parental GBM cells treated with the CM derived from Spg-miR142-3p-transfected cells expressed high levels of Nestin (a neuroprogenitor marker) and phosphorylated STAT3 (a downstream molecule of IL-6 signaling); conversely, the addition of IL-6-nAb suppressed expression of both of these proteins in vivo (Figure 4G). Phosphorylated STAT3 was also increased by Spg-miR142-3p and suppressed by shIL-6 or IL-6-nAb in CM-treated parental GBM cells (Figure S5E). These data, combined with our previous results, demonstrate that miR142-3p negatively regulates the IL-6-STAT3 pathway and suggest the involvement of IL-6-STAT3 signaling in the miR142-3p and IL-6 regulatory loop in GBM-CSC properties.

miR142-3p Targets the HMGA2 3′ UTR and Regulates the Binding of HMGA2 to the Sox2 Promoter

HMGA2, a member of the high-mobility group AT-hook protein family, was reported to bind directly to the cyclin B2 and E2F1 promoter and contribute to pituitary tumorigenesis (De Martino et al., 2009). Following our discovery that HMGA2 is a potential mediator of miR142-3p, we performed a series of HMGA2 3′ UTR reporter assays and showed that miR142-3p directly targeted HMGA2 at its 3′ UTR through a specific sequence (Figures 5A and S6A). Ectopic expression of miR142-3p expression inhibited the protein levels of endogenous HMGA2 and IL-6 in NBE-GBMs (Figures 5B and S6B). In contrast, knocking down miR142-3p (Spg-miR142-3p) enhanced HMGA2 and IL-6 levels in parental GBM cells (Figure 5B). Moreover, silencing of HMGA2 in Spg-miR142-3p-transfected parental GBMs suppressed the growth of xenografted tumors in immunocompromised mice (Figure 5C). Immunohistochemistry further confirmed that the percentage of HMGA2-positive cells was increased by Spg-miR142-3p but suppressed by shHMGA2 in the xenografted tumors (Figures 5D and 5E). Concurrently, HMGA2 knockdown also suppressed the mRNA levels of glioma stem cell markers Oligo2 and Sox2 in the Spg-miR142-3p-GBM-xenografted tumors (Figure 5F).

Sox2, a signature of brain tumor-derived stem cells, is critical for maintaining their self-renewing and stemness properties (Gangemi et al., 2009). We showed that overexpression of HMGA2 in parental GBM cells increased the protein levels of Sox2, and knockdown of HMGA2 in NBE-GBM cells suppressed Sox2, but not vice versa (Figures 5G and S6C). Luciferase reporter assay indicated that HMGA2 enhanced Sox2 promoter activity, while deletion or mutation of the AT-hook region 2 in the Sox2 promoter prevented HMGA2 from activating the Sox2 promoter (Figure 5H). ChIP assay demonstrated that the Sox2 promoter containing wild-type, but not mutant AT-hook region 2 (mutation or deletion), coprecipitated with HMGA2, indicating that the AT-hook region 2 is the dominant HMGA2-responsive element (Figure 5I). The interaction of HMGA2 with the Sox2 promoter was further validated by an electrophoretic mobility shift assay (EMSA; Figure 5J). Moreover, the HMGA2-Sox2 pathway was negatively regulated by miR142-3p (Figure S6D), which enhances Sox2-related stemness (Figure S6E) and tumorigenesis (Figure S6F) in GBM.

Clinical Significance of IL-6, HMGA2, SOX2 Expression, and Promoter Methylation of miR142-3p

Based on our in vitro and in vivo data, IL-6, HMGA2, and SOX2 play critical roles in the miR142-3p-dependent regulatory circuit in GBM. Therefore, we investigated the clinical significance of the IL-6, HMGA2, and SOX2 protein expression levels in the survival outcome of GBM patients collected from multiple centers in Taiwan (Table S1). Two independent groups of samples (group A: IL-6^{hi}HMGA2^{hi}Sox2^{hi}, n = 69; group B: IL-6^{lo}HMGA2^{lo}Sox2^{lo}, n = 66; Table S2) were determined by immunohistochemical (IHC) staining and subjected to a Kaplan-Meier survival analysis. GBM patients with the expression pattern of IL-6^{hi}HMGA2^{hi}Sox2^{hi} had shorter overall survival compared with those with IL-6^{lo}HMGA2^{lo}Sox2^{lo} (Figure 6B; $p < 0.001$). In support of the closely related relationship of the three molecules in clinical specimens, we demonstrated a colocalization between HMGA2 and IL-6 (Figure 6C, left) as well as HMGA2 and Sox2 (Figure 6C, right) in the same foci of GBM tissues from patients with IL-6^{hi}HMGA2^{hi}Sox2^{hi}.

Next, we wanted to evaluate the methylation status of miR142-3p promoter in these 135 clinical GBM specimens. Due to the high heterogeneity of GBM specimens, which contain a considerable proportion of noncancerous stromal cells, we applied laser capture microdissection (LCM) (van Hoesel et al., 2013) to specifically dissect the tumor tissues from 135 deparaffinized GBM samples (Figure 6D). With 6 pairs of dissected tumor (T) and nontumor (stroma; S) tissue samples, the results from methylation-specific PCR analysis showed that the miR142-3p promoter was highly methylated in all tumor tissues and less methylated in stromal parts (Figure 6E). Interestingly, unmethylated (UnMeth) miR142-3p promoter was detected in 60 out of the 66 IL-6^{lo}HMGA2^{lo}Sox2^{lo} specimens, whereas hypermethylated (Meth) miR142-3p promoter was detected in 56 out of the 69 IL-6^{hi}HMGA2^{hi}Sox2^{hi} specimens (Figure 6F). These results suggest a correlation between hypermethylated miR142-3p promoter and IL-6^{hi}HMGA2^{hi}Sox2^{hi} expression pattern in clinical samples.

We then subjected 73 unmethylated miR142-3p promoter samples (60 of IL-6^{lo}HMGA2^{lo}Sox2^{lo} and 13 of IL-6^{hi}HMGA2^{hi}Sox2^{hi}) and 62 methylated miR142-3p promoter samples (6 of IL-6^{lo}HMGA2^{lo}Sox2^{lo} and 56 of IL-6^{hi}HMGA2^{hi}Sox2^{hi}) to a Kaplan-Meier survival analysis and showed that patients with hypermethylated miR142-3p promoter had worse survival outcome than those with unmethylated promoter (Figure 6G; $p < 0.001$). In particular, patients expressing hypermethylated

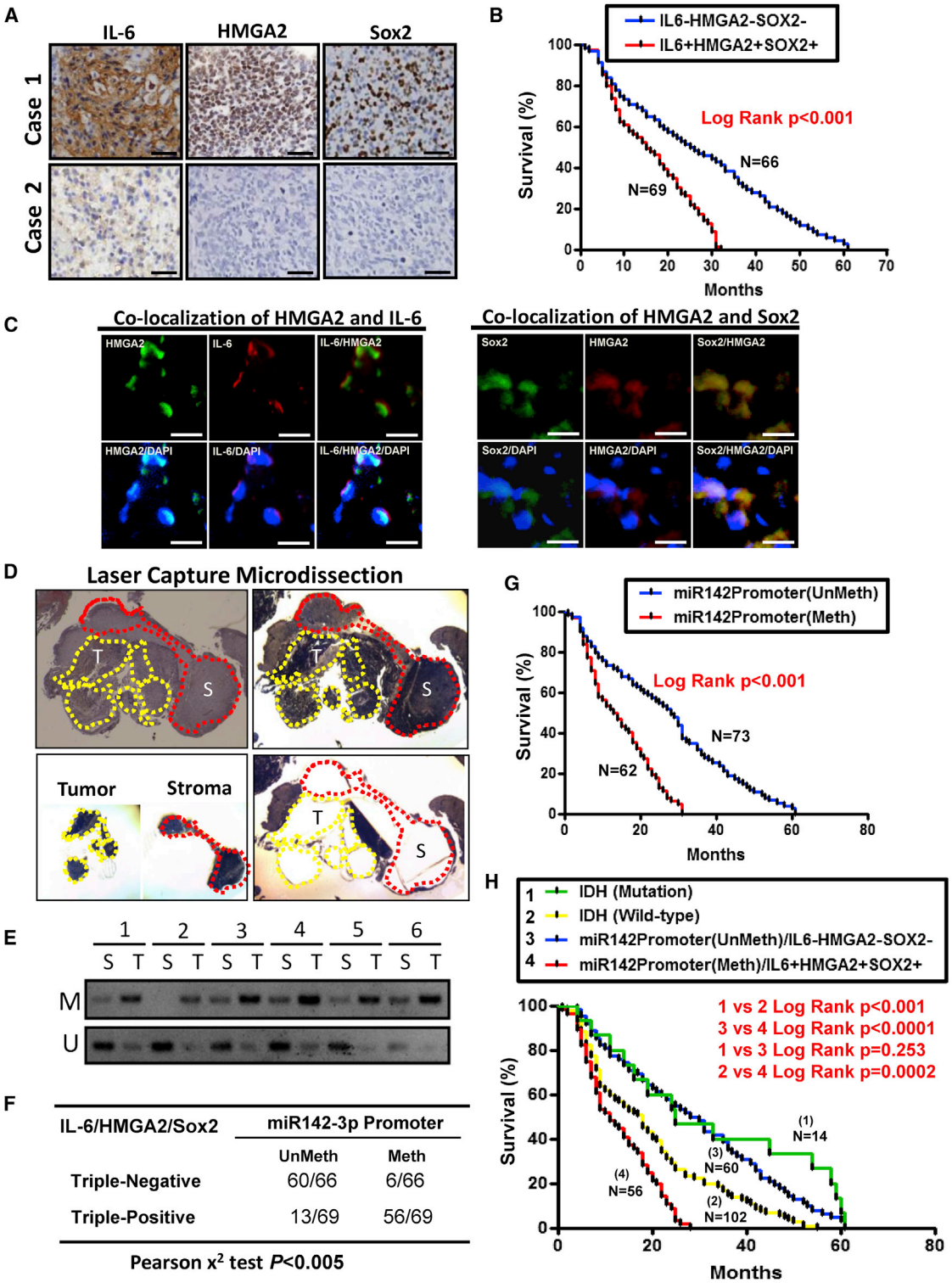

Figure 6. Clinical Significance of the Protein Expression of IL-6/HMGA2/Sox2 and Hypermethylation of The miR142-3p Promoter

(A) Representative images of IHC staining of IL-6, HMGA2, and Sox2 in two GBM groups. Case 1 (group A), coexpression of IL-6, HMGA2, and Sox2 (triple positive). Case 2 (group B), noncoexpression (triple negative).

(B) Overall survival periods of GBM patients in group A (coexpression of IL-6/HMGA2/Sox2; n = 69) versus group B (noncoexpression; n = 66) in a total of 135 GBM patients. p value was estimated by a log-rank test.

(legend continued on next page)

miR142-3p promoter plus a protein signature of IL-6^{hi}HMGA2^{hi} Sox2^{hi} presented a poor survival outcome (Figure 6H), while those expressing unmethylated promoter plus an IL-6^{lo} HMGA2^{lo}Sox2^{lo} signature presented a better outcome; the statistical significance between the two groups is greater than when using either miR142-3p promoter or IL-6/HMGA2/Sox2 as prognosis marker (Figure 6H; Table S3; $p < 0.0001$). IDH1^{R132} mutation has also been suggested as an important marker for GBM classification and methylation status, glioma CpG island methylator phenotype, or G-CIMP (Noushmehr et al., 2010; Yan et al., 2009). Using the same data sets as described in Figure 6H, we also evaluated the IDH1^{R132} mutation. Similar to previous studies (Turcan et al., 2012), we showed that GBM patients with IDH1 mutation had a better survival outcome ($p < 0.001$). Notably, in our defined 116 patient samples from multiple cancer centers in Taiwan (Table S3), patients with hypermethylated miR142-3p promoter plus a protein signature of IL-6^{hi}HMGA2^{hi}Sox2^{hi} showed a worse survival outcome than those with wild-type IDH1 (Figure 6H; $p = 0.0002$). Collectively, these results suggest that hypermethylated miR142-3p promoter with an IL-6^{hi}HMGA2^{hi}Sox2^{hi} signature may be used as a predictor for disease progression and poor clinical outcome in GBM patients.

Orthotopic Delivery of miR142-3p Mimics Inhibits Tumorigenicity and Blocks IL-6, HMGA2, and SOX2 Expression in GBM-Transplanted Severe Combined Immunodeficiency Mice

To examine the therapeutic potential of miR142-3p in GBM tumor progression, synthetic hsa-miR-142-3p and miRNA precursor control mimic were used (Takeshita et al., 2010). We first tested the microRNA mimics targeting fidelities by examining several miR142-3p-targeting genes in GBM-NBE cells, with results showing a consistent decrease in the expression of predicted miR142-3p-targeting genes, including Rac1, HMGA2, and Sox2. In addition, there was no effect induced by miR142-3p mimics on GADPH or β-actin expression levels (Figure S7A). The treatment of miR142-3p mimics effectively suppressed the ability of cell proliferation or tumor growth of GBM-CSC in vitro (Figure S7B). We then evaluated the therapeutic potential of synthetic miR142-3p in GBM tumor-bearing mice. GFP-labeled NBE-cultured GBMs were first injected into the stratum of mice brains (Yang et al., 2012) before injecting control, atelocollagen only, NC plus atelocollagen, or synthetic-miR-142-3p plus atelocollagen into the xenografted tumor site. In vivo GFP imaging showed that injection of synthetic-miR-142-3p plus atelocollagen, but not NC plus atelocollagen, reduced the size of GBM tumor xenografts derived from two NBE-cultured GBM clones (Figures 7A and 7B). Hematoxylin and eosin staining of these tumor sections showed diffused vascular-like formation in tumors injected with NC plus atelocollagen, but not in those injected with synthetic-miR-142-3p plus atelocollagen (Figure 7B, left). To validate the therapeutic utility of miR142-3p in GBM-CSC, GBM-CD133^{+} and GBM-SP^{+} cells were transplanted in immunocompromised mice, followed by treatment of synthetic miR142-3p with atelocollagen. Delivery of synthetic miR142-3p reduced CSC-derived tumor growth (Figures 7C and 7D). Notably, IHC showed that the number of HMGA2^{+}, Sox2^{+}, and IL-6^{+} cells in the xenografted tumors derived from GBM-NBE, GBM-CD133^{+}, and GBM-SP^{+} were largely suppressed by the delivery of synthetic-miR-142-3p plus atelocollagen, but not NC plus atelocollagen (Figures 7B–7D and S7C). Furthermore, we demonstrated that synthetic-miR142-3p plus atelocollagen significantly improved the survival rate of mice bearing GBM tumor xenografts that developed from transplanted NBE-culturedGBM or Spg-miR142-3p-transfected parental GBM cells (Figures 7B and S7D). Together, these results indicate that miR142-3p is a potential therapeutic molecule suppressing the miR142-3p-related HMGA2/Sox2/IL-6 axis in GBM, and delivery of synthetic miR-142-3p with atelocollagen reduces tumor growth of GBM with stem-like properties and prolongs animal survival.

DISCUSSION

Aberrant epigenetic regulations have been implicated in malignant transformation and stemness acquisition of cancer. Kim et al. (2013) showed that EZH2 binds to and methylates STAT3, leading to enhanced phosphorylation and activity of STAT3 in GBM stem-like cells. DNMT1 is well known for its role in maintaining stemness properties in both somatic stem cells and CSCs (Foran et al., 2010; Sen et al., 2010); its involvement in GBM-CSCs, however, is not clear. We showed in this study that through suppression of miR142-3p expression, DNMT1 enhances GBM-CSC-like properties, shifting the cell characteristics from non-CSC to CSC. This regulation involves a competition between DNMT1-mediated methylation and Sp1 binding on the Sp1-binding motif in the promoter of miR142-3p. Once the Sp1 motif is hypermethylated, it is no longer able to associate with the miR142-3p promoter, resulting in decreased miR142-3p transcription. Similar competition between Sp1 binding and the methylation mechanism on the gene promoter has also been reported in the regulation of CD133 (Gopisetty et al., 2013). We further demonstrated that this regulatory mechanism can be modulated in an autocrine and paracrine manner: IL-6, a downstream target of miR142-3p,

(C) Immunofluorescent staining of HMGA2, Sox2, and IL-6 protein localization in GBM patient (group A) tissues.
(D) LCM was performed to capture the tumorous (T) and stromal (S) tissues from deparaffinized, hematoxylin-stained GBM samples. A representative slide capturing GBM tumor and stromal tissue is shown.
(E) Methylation status of the miR142-3p promoter in six pairs of LCM-captured tumorous and stromal tissue samples by methylation-specific PCR (MSP) analysis.
(F) Kaplan-Meier survival analysis between the hypermethylated group (n = 62) and nonmethylated group (n = 73) of the miR142-3p promoter in GBM patients.
(G) Statistical analysis of the correlation between hypermethylated and unmethylated miR142-3p promoter.
(H) Survival analysis between hypermethylated miR142-3p promoter plus HMGA2^{hi}/Sox2^{hi}/IL-6^{hi} (n = 56) and unmethylated miR142-3p promoter plus HMGA2^{lo}/Sox2^{lo}/IL-6^{lo} (n = 60) in patient tissue samples. $p < 0.0001$. The same data set was further analyzed according to the IDH mutation status (IDH mutation, n = 14; IDH wild-type, n = 102) and correlated with the patient survival outcome. $p < 0.001$.
See also Figure S5 and Tables S1–S3.

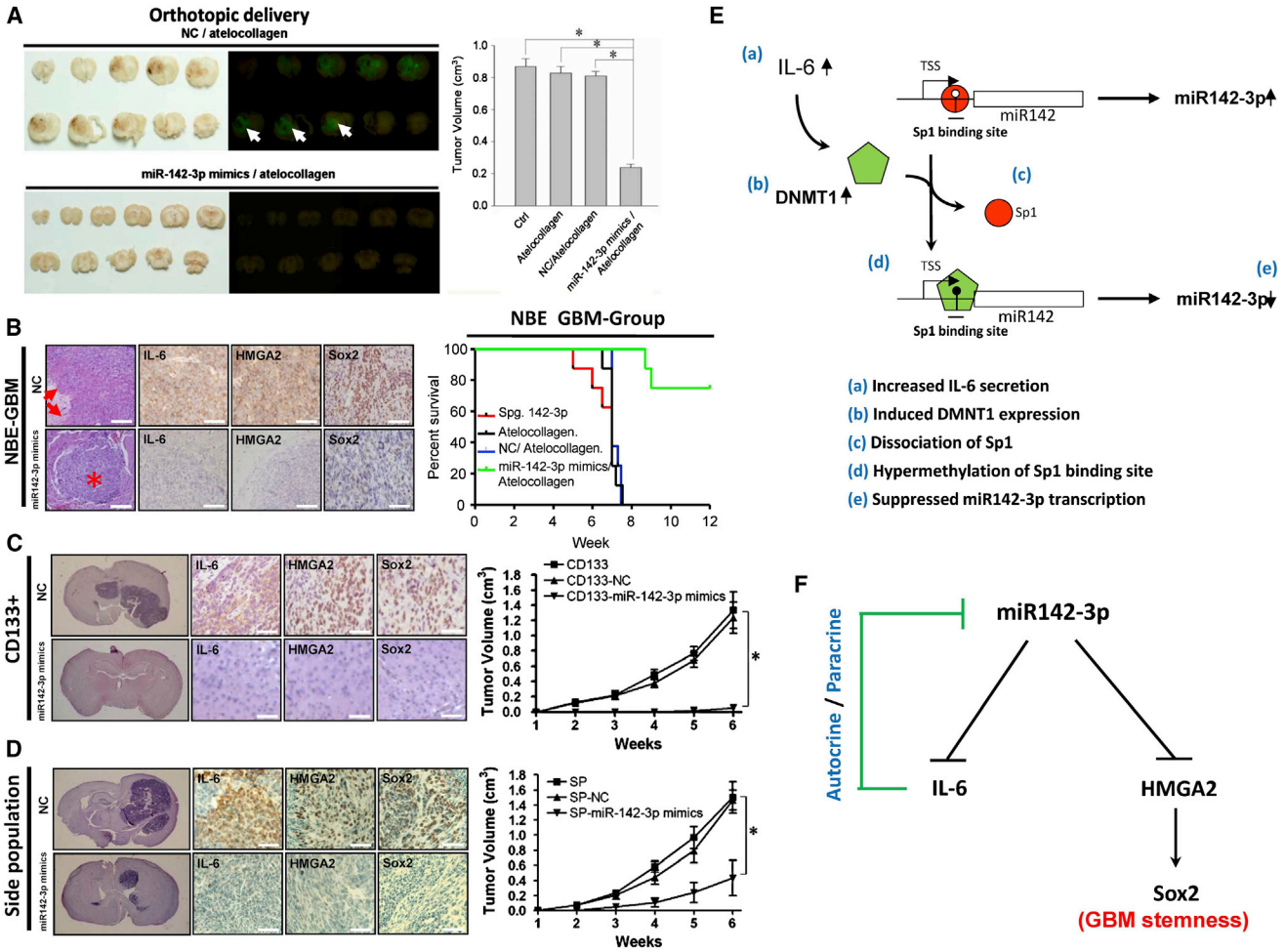

Figure 7. Orthotopic Delivery of Synthetic miR142-3p Presents a Therapeutic Effect in Immunocompromised Mice Transplanted with GBM-CSCs

(A) Representative images of tumors isolated from immunocompromised mice transplanted with GFP-labeled GBM cells treated with atelocollagen only, NC/atelocollagen, or synthetic-miR-142-3p/atelocollagen. Whole brains were isolated, and the GFP signals (arrows), which indicate the tumor locus, were photographed and quantified (right).

(B) IHC staining of IL-6, HMGA2, and Sox2 in NBE-GBM-derived tumor specimens from mice treated with or without miR142-3p. The survival rates of mice were observed for up to 12 weeks to evaluate the effect of miR142-3p on survival in vivo. *tumor site.

(C and D) Cells (GBM-CD133$^+$ [C] and GBM-SP$^+$ [D]) were transplanted in immunocompromised mice and treated with NC/atelocollagen or synthetic-miR-142-3p/atelocollagen. The growth of CD133$^+$- or GBM-SP$^+$-derived tumors was monitored by MRI, and IL-6/HMGA2/Sox2 expression in specimens was detected by IHC.

(E) Illustration showing that IL-6 inhibits miR142-3p expression by mediating DNMT1-dependent methylation of the Sp1-bining site of the miR142-3p promoter.

(F) Schematic of the autocrine- and paracrine-mediated regulation of miR142-3p/IL-6 and the miR142-3p/HMGA2/Sox2 circuit.

Error bars in (A), (C), and (D) indicate mean ± SEM; n = 6. *$p < 0.01$. See also Figure S7.

suppresses miR142-3p expression by increasing DNMT1 expression and DNMT1-mediated methylation on the miR142-3p promoter, abolishing Sp1-dependent enhancement of miR142-3p transcription. Our finding is in line with a previous report showing that IL-6 upregulates DNMT1-mediated colon tumorigenesis (Foran et al., 2010) and revealed an IL-6/miR142-3p feedback circuit in GBM (Figures 7E and 7F).

Accumulating evidence has emphasized important roles of miRNAs in regulating CSC-like properties (Silber et al., 2008). We consistently observed lower miR142-3p levels in NBE-GBM, GBM-CD133$^+$, and GBM-SP$^+$ subsets of cells, which are enriched with CSC-like tumor-propagating cells. In these subsets, we identified HMGA2 as a direct target of miR142-3p and Sox2 as a transcriptional target of HMGA2. Through suppressing HMGA2 levels, miR142-3p represses Sox2 and Sox2-mediated cancer stemness (Figure 7F). Manipulating miR142-3p expression significantly altered the stem-like properties of GBM-CD133$^+$ and GBM-SP$^+$ subsets and the size of their tumor growth. In contrast, SPONGE-miR142-3p increased the in vivo tumor-propagating ability of primary GBM by 100- to 10,000-fold. Additionally, IDH1 mutation has been well established as a marker to predict GBM patient survival (Turcan

et al., 2012), which was also validated in our study. However, hypermethylated miR142-3p promoter plus upregulated IL-6/HMGA2/SOX2 protein levels in GBM were highly correlated with a poor outcome of GBM patient survival and more significant in predicting GBM prognosis, indicating that the miR142-3p signaling could serve as an alternative marker for GBM prognosis.

The miR142-3p/HMGA2/Sox2 signaling reveals a crucial role of miR142-3p in GBM propagation: suppressing miR142-3p is crucial for GBM-CSCs to acquire and maintain tumor stem-like properties. Our data demonstrate a cytokine-mediated epigenetic regulatory mechanism on the miR142-3p-dependent circuit in GBM malignancy and that inducing miR142-3p expression level has the potential to specifically target the GBM-CSC.

EXPERIMENTAL PROCEDURES

Cells and Preparation

All experimental procedures were performed as described previously (Yang et al., 2012) and in the Supplemental Information. The dissociated GBM cells were suspended with NBE media consisting of neurobasal media (Invitrogen), N2/B27 supplements (Invitrogen), human recombinant bFGF/EGF (20 ng/ml; R&D Systems), or serum media consisting of DMEM/F12 media (Invitrogen) with 10% FBS (HyClone).

Methylation-Specific PCR and Sequencing

Genomic DNA (1 μg) from patient samples was extracted and subjected to bisulfite conversion, and the converted genomic DNA was subjected to two PCR amplifications. The primers used are listed in the Supplemental Information.

Statistical Analysis

A Student's t test or ANOVA test was used to compare the continuous variables between groups, as appropriate. The chi-square test or Fisher's exact test was used to compare the categorical variables. Survival was estimated by the Kaplan-Meier method and compared by the log-rank test. A p value < 0.05 was considered statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.molcel.2013.11.009.

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REFERENCES

Bostick, M., Kim, J.K., Estève, P.O., Clark, A., Pradhan, S., and Jacobsen, S.E. (2007). UHRF1 plays a role in maintaining DNA methylation in mammalian cells. Science *317*, 1760–1764.

Braconi, C., Huang, N., and Patel, T. (2010). MicroRNA-dependent regulation of DNA methyltransferase-1 and tumor suppressor gene expression by interleukin-6 in human malignant cholangiocytes. Hepatology *51*, 881–890.

Chen, C.Z., Li, L., Lodish, H.F., and Bartel, D.P. (2004). MicroRNAs modulate hematopoietic lineage differentiation. Science *303*, 83–86.

De Martino, I., Visone, R., Wierinckx, A., Palmieri, D., Ferraro, A., Cappabianca, P., Chiappetta, G., Forzati, F., Lombardi, G., Colao, A., et al. (2009). HMGA proteins up-regulate CCNB2 gene in mouse and human pituitary adenomas. Cancer Res. *69*, 1844–1850.

Deng, G., Song, G.A., Pong, E., Sleisenger, M., and Kim, Y.S. (2004). Promoter methylation inhibits APC gene expression by causing changes in chromatin conformation and interfering with the binding of transcription factor CCAAT-binding factor. Cancer Res. *64*, 2692–2698.

Estève, P.O., Chin, H.G., and Pradhan, S. (2005). Human maintenance DNA (cytosine-5)-methyltransferase and p53 modulate expression of p53-repressed promoters. Proc. Natl. Acad. Sci. USA *102*, 1000–1005.

Estève, P.O., Chin, H.G., and Pradhan, S. (2007). Molecular mechanisms of transactivation and doxorubicin-mediated repression of survivin gene in cancer cells. J. Biol. Chem. *282*, 2615–2625.

Foran, E., Garrity-Park, M.M., Mureau, C., Newell, J., Smyrk, T.C., Limburg, P.J., and Egan, L.J. (2010). Upregulation of DNA methyltransferase-mediated gene silencing, anchorage-independent growth, and migration of colon cancer cells by interleukin-6. Mol. Cancer Res. *8*, 471–481.

Gangemi, R.M., Griffero, F., Marubbi, D., Perera, M., Capra, M.C., Malatesta, P., Ravetti, G.L., Zona, G.L., Daga, A., and Corte, G. (2009). SOX2 silencing in glioblastoma tumor-initiating cells causes stop of proliferation and loss of tumorigenicity. Stem Cells *27*, 40–48.

Gillies, J.K., and Lorimer, I.A. (2007). Regulation of p27Kip1 by miRNA 221/222 in glioblastoma. Cell Cycle *6*, 2005–2009.

Gopisetty, G., Xu, J., Sampath, D., Colman, H., and Puduvalli, V.K. (2013). Epigenetic regulation of CD133/PROM1 expression in glioma stem cells by Sp1/myc and promoter methylation. Oncogene *32*, 3119–3129.

Guan, H., Cai, J., Zhang, N., Wu, J., Yuan, J., Li, J., and Li, M. (2012). Sp1 is upregulated in human glioma, promotes MMP-2-mediated cell invasion and predicts poor clinical outcome. Int. J. Cancer *130*, 593–601.

Hermansen, S.K., and Kristensen, B.W. (2013). MicroRNA biomarkers in glioblastoma. J. Neurooncol. *114*, 13–23.

Hervouet, E., Vallette, F.M., and Cartron, P.F. (2010). Dnmt1/Transcription factor interactions: an alternative mechanism of DNA methylation inheritance. Genes Cancer *1*, 434–443.

Iliopoulos, D., Hirsch, H.A., and Struhl, K. (2009). An epigenetic switch involving NF-kappaB, Lin28, Let-7 MicroRNA, and IL6 links inflammation to cell transformation. Cell *139*, 693–706.

Inda, M.M., Bonavia, R., Mukasa, A., Narita, Y., Sah, D.W., Vandenberg, S., Brennan, C., Johns, T.G., Bachoo, R., Hadwiger, P., et al. (2010). Tumor heterogeneity is an active process maintained by a mutant EGFR-induced cytokine circuit in glioblastoma. Genes Dev. *24*, 1731–1745.

Kim, E., Kim, M., Woo, D.H., Shin, Y., Shin, J., Chang, N., Oh, Y.T., Kim, H., Rheey, J., Nakano, I., et al. (2013). Phosphorylation of EZH2 activates STAT3 signaling via STAT3 methylation and promotes tumorigenicity of glioblastoma stem-like cells. Cancer Cell *23*, 839–852.

Korkaya, H., Kim, G.I., Davis, A., Malik, F., Henry, N.L., Ithimakin, S., Quraishi, A.A., Tawakkol, N., D'Angelo, R., Paulson, A.K., et al. (2012). Activation of an IL6 inflammatory loop mediates trastuzumab resistance in HER2+ breast cancer by expanding the cancer stem cell population. Mol. Cell *47*, 570–584.

Lee, J., Kotliarova, S., Kotliarov, Y., Li, A., Su, Q., Donin, N.M., Pastorino, S., Purow, B.W., Christopher, N., Zhang, W., et al. (2006). Tumor stem cells derived from glioblastomas cultured in bFGF and EGF more closely mirror

the phenotype and genotype of primary tumors than do serum-cultured cell lines. Cancer Cell *9*, 391–403.

Meng, F., Wehbe-Janek, H., Henson, R., Smith, H., and Patel, T. (2008). Epigenetic regulation of microRNA-370 by interleukin-6 in malignant human cholangiocytes. Oncogene *27*, 378–386.

Møller, H.G., Rasmussen, A.P., Andersen, H.H., Johnsen, K.B., Henriksen, M., and Duroux, M. (2013). A systematic review of microRNA in glioblastoma multiforme: micro-modulators in the mesenchymal mode of migration and invasion. Mol. Neurobiol. *47*, 131–144.

Noushmehr, H., Weisenberger, D.J., Diefes, K., Phillips, H.S., Pujara, K., Berman, B.P., Pan, F., Pelloski, C.E., Sulman, E.P., Bhat, K.P., et al.; Cancer Genome Atlas Research Network (2010). Identification of a CpG island methylator phenotype that defines a distinct subgroup of glioma. Cancer Cell *17*, 510–522.

Rokavec, M., Wu, W., and Luo, J.L. (2012). IL6-mediated suppression of miR-200c directs constitutive activation of inflammatory signaling circuit driving transformation and tumorigenesis. Mol. Cell *45*, 777–789.

Sansone, P., Storci, G., Tavolari, S., Guarnieri, T., Giovannini, C., Taffurelli, M., Ceccarelli, C., Santini, D., Paterini, P., Marcu, K.B., et al. (2007). IL-6 triggers malignant features in mammospheres from human ductal breast carcinoma and normal mammary gland. J. Clin. Invest. *117*, 3988–4002.

Sen, G.L., Reuter, J.A., Webster, D.E., Zhu, L., and Khavari, P.A. (2010). DNMT1 maintains progenitor function in self-renewing somatic tissue. Nature *463*, 563–567.

Sharif, J., Muto, M., Takebayashi, S., Suetake, I., Iwamatsu, A., Endo, T.A., Shinga, J., Mizutani-Koseki, Y., Toyoda, T., Okamura, K., et al. (2007). The SRA protein Np95 mediates epigenetic inheritance by recruiting Dnmt1 to methylated DNA. Nature *450*, 908–912.

Sharma, S., Liu, J., Wei, J., Yuan, H., Zhang, T., and Bishopric, N.H. (2012). Repression of miR-142 by p300 and MAPK is required for survival signalling via gp130 during adaptive hypertrophy. EMBO Mol Med *4*, 617–632.

Silber, J., Lim, D.A., Petritsch, C., Persson, A.I., Maunakea, A.K., Yu, M., Vandenberg, S.R., Ginzinger, D.G., James, C.D., Costello, J.F., et al. (2008). miR-124 and miR-137 inhibit proliferation of glioblastoma multiforme cells and induce differentiation of brain tumor stem cells. BMC Med. *6*, 14.

Straussman, R., Morikawa, T., Shee, K., Barzily-Rokni, M., Qian, Z.R., Du, J., Davis, A., Mongare, M.M., Gould, J., Frederick, D.T., et al. (2012). Tumour micro-environment elicits innate resistance to RAF inhibitors through HGF secretion. Nature *487*, 500–504.

Stupp, R., Mason, W.P., van den Bent, M.J., Weller, M., Fisher, B., Taphoorn, M.J., Belanger, K., Brandes, A.A., Marosi, C., Bogdahn, U., et al.; European Organisation for Research and Treatment of Cancer Brain Tumor and Radiotherapy Groups; National Cancer Institute of Canada Clinical Trials Group (2005). Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. N. Engl. J. Med. *352*, 987–996.

Stupp, R., Hegi, M.E., Gilbert, M.R., and Chakravarti, A. (2007). Chemoradiotherapy in malignant glioma: standard of care and future directions. J. Clin. Oncol. *25*, 4127–4136.

Sun, Y., Varambally, S., Maher, C.A., Cao, Q., Chockley, P., Toubai, T., Malter, C., Nieves, E., Tawara, I., Wang, Y., et al. (2011). Targeting of microRNA-142-3p in dendritic cells regulates endotoxin-induced mortality. Blood *117*, 6172–6183.

Sun, Y., Sun, J., Tomomi, T., Nieves, E., Mathewson, N., Tamaki, H., Evers, R., and Reddy, P. (2013). PU.1-dependent transcriptional regulation of miR-142 contributes to its hematopoietic cell-specific expression and modulation of IL-6. J. Immunol. *190*, 4005–4013.

Takeshita, F., Patrawala, L., Osaki, M., Takahashi, R.U., Yamamoto, Y., Kosaka, N., Kawamata, M., Kelnar, K., Bader, A.G., Brown, D., and Ochiya, T. (2010). Systemic delivery of synthetic microRNA-16 inhibits the growth of metastatic prostate tumors via downregulation of multiple cell-cycle genes. Mol. Ther. *18*, 181–187.

Turcan, S., Rohle, D., Goenka, A., Walsh, L.A., Fang, F., Yilmaz, E., Campos, C., Fabius, A.W., Lu, C., Ward, P.S., et al. (2012). IDH1 mutation is sufficient to establish the glioma hypermethylator phenotype. Nature *483*, 479–483.

van Hoesel, A.Q., Sato, Y., Elashoff, D.A., Turner, R.R., Giuliano, A.E., Shamonki, J.M., Kuppen, P.J., van de Velde, C.J., and Hoon, D.S. (2013). Assessment of DNA methylation status in early stages of breast cancer development. Br. J. Cancer *108*, 2033–2038.

Wang, X.S., Gong, J.N., Yu, J., Wang, F., Zhang, X.H., Yin, X.L., Tan, Z.Q., Luo, Z.M., Yang, G.H., Shen, C., and Zhang, J.W. (2012). MicroRNA-29a and microRNA-142-3p are regulators of myeloid differentiation and acute myeloid leukemia. Blood *119*, 4992–5004.

Weissenberger, J., Loeffler, S., Kappeler, A., Kopf, M., Lukes, A., Afanasieva, T.A., Aguzzi, A., and Weis, J. (2004). IL-6 is required for glioma development in a mouse model. Oncogene *23*, 3308–3316.

Wu, L., Cai, C., Wang, X., Liu, M., Li, X., and Tang, H. (2011). MicroRNA-142-3p, a new regulator of RAC1, suppresses the migration and invasion of hepatocellular carcinoma cells. FEBS Lett. *585*, 1322–1330.

Yan, H., Parsons, D.W., Jin, G., McLendon, R., Rasheed, B.A., Yuan, W., Kos, I., Batinic-Haberle, I., Jones, S., Riggins, G.J., et al. (2009). IDH1 and IDH2 mutations in gliomas. N. Engl. J. Med. *360*, 765–773.

Yang, Y.P., Chien, Y., Chiou, G.Y., Cherng, J.Y., Wang, M.L., Lo, W.L., Chang, Y.L., Huang, P.I., Chen, Y.W., Shih, Y.H., et al. (2012). Inhibition of cancer stem cell-like properties and reduced chemoradioresistance of glioblastoma using microRNA145 with cationic polyurethane-short branch PEI. Biomaterials *33*, 1462–1476.