Silencing of Thrombospondin-1 Is Critical for Myc-Induced Metastatic Phenotypes in Medulloblastoma

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Abstract

Mechanisms by which c-Myc (Myc) amplification confers aggressive medulloblastoma phenotypes are poorly defined. Here, we show using orthotopic models that high Myc expression promotes cell migration/invasion and induces metastatic tumors, which recapitulate aggressive histologic features of Myc-amplified primary human medulloblastoma. Using ChIP-chip analysis, we identified cell migration and adhesion genes, including Tsp-1/THBS1, ING4, PVRL3, and PPAP2B, as Myc-bound loci in medulloblastoma cells. Expression of Tsp-1 was most consistently and robustly diminished in medulloblastoma cell lines and primary human tumors with high Myc expression (r = 101, P = 0.032). Strikingly, stable Tsp-1 expression significantly attenuated in vitro transformation and invasive/migratory properties of high Myc-expressing medulloblastoma cells without altering cell proliferation, whereas RNA interference–mediated Myc knockdown was consistently accompanied by increased Tsp-1 levels and reduced cell migration and invasion in medulloblastoma cells. Chromatin immunoprecipitation (ChIP) assays revealed colocalization of Myc and obligate partner Max and correlated diminished RNA polymerase II occupancy (∼3-fold decrease, P < 0.01) with increased Myc binding at a core Tsp-1 promoter. Reporter gene and/or gel shift assays confirmed direct repression of Tsp-1 transcription by Myc and also identified JPO2, a Myc interactor associated with metastatic medulloblastoma, as a cofactor in Myc-mediated Tsp-1 repression. These findings indicate the Myc-regulatory network targets Tsp-1 via multiple mechanisms in medulloblastoma transformation, and highlight a novel critical role for Tsp-1 in Myc-mediated aggressive medulloblastoma phenotypes. Cancer Res; 70(20); 8199–210. ©2010 AACR.

Introduction

Medulloblastoma represents approximately one fifth of all pediatric brain tumors and is the most common malignant brain tumor in children. Although survival has improved substantially, ∼25% to 30% of children with medulloblastoma will have treatment-refractory recurrent disease. A better understanding of biological mechanisms that drive medulloblastoma progression/recurrence is needed for further improvement in survival.

In addition to tumor metastasis, biological features linked to poorer prognosis in medulloblastoma include tumor grade defined by histopathologic features of anaplasia, loss of chr17p or gain of 17q, and activation of the PDGFRα, Erbb2, or Myc family of oncogenes (1–4). Substantial evidence indicates important roles for Myc oncogenes in medulloblastoma progression; specifically, c-Myc or N-Myc gene amplification (5–7) and high Myc mRNA or protein expression correlate with increased risk of treatment failure and poor survival (8, 9). Amplification of c-Myc or N-Myc, seen in 10% to 20% of all primary medulloblastoma, is reported in up to 50% of medulloblastoma with aggressive large cell anaplastic histology (3, 7, 10), and experimental studies show that high c-Myc (hereon referred to as Myc) expression can promote anaplastic tumor histology (11). Most importantly, Myc amplification may be acquired in recurrent medulloblastoma (12).

Oncogenic properties of Myc proteins depend on their transcriptional regulatory functions. Myc activates transcription by dimerizing with obligate partner Max to bind E-box (CANNTG) motifs in target genes. Myc-mediated transcriptional repression is poorly defined and does not depend on direct Myc E-box interactions (13–15) but may occur via inhibitory interactions of Myc with DNA-binding transcriptional activators such as Miz-1 (16). Consistent with its pleiotropic properties, Myc can induce widespread gene expression changes by direct regulation of diverse...
cellular targets, including microRNA loci, as well as global alteration of chromatin structure (10, 17). Factors that influence Myc target selection and oncogenic effects are poorly understood and may be cell context and cell type dependent (18). Myc is known to promote tumor formation and metastases by targeting diverse cellular processes, including metabolism, differentiation, cell adhesion, and angiogenesis, in many advanced human neoplasms (14, 18–20); however, biological and molecular mechanisms by which Myc confers aggressive medulloblastoma phenotypes remain to be elucidated.

In this study, we sought to define Myc-transforming functions most relevant to medulloblastoma progression by developing Myc-driven orthotopic tumor models, which recapitulate histologic and clinical features of primary metastatic medulloblastoma, and interrogating them with ChIP-chip analysis to uncover Myc effector genes critical in medulloblastoma transformation.

Materials and Methods

Cell lines

Medulloblastoma cell lines UW228, UW426 (gifts from Dr. Silber, University of Washington), and Daoy [American Type Culture Collection (ATCC)] were maintained in α-MEM; D341, D283, D425, and D458 cells (gifts from Dr. Bigner, Duke University) were maintained in calcium carbonate medium (Life Technologies); and ONS76 and SKNBE(2) (ATCC) cells were maintained in Eagle’s MEM/F12. All media contained 10% fetal bovine serum (FBS). HO15.19 (ATCC) cells were maintained in Eagle’s MEM/F12. All media were supplemented with 10% fetal bovine serum (FBS). Myc knockdown was confirmed by quantitative reverse transcriptase polymerase chain reaction analysis (qPCR) using primers that span the minimal Myc-binding region (N262), Max, RNA polymerase II, SP1, or Miz-1 antibodies

Protein and RNA analyses

Cell lysates were prepared using EBC buffer (24) and immunoblotted with antibodies to Myc (in-house 9E10 monoclonal), Tsp-1 (Abcam), β-actin, or α-tubulin (Sigma-Aldrich).

For gene expression analysis, cDNAs were generated using standard methods and used in semiquantitative or quantitative PCR (qPCR) analysis (SYBR Green PCR kit, Invitrogen; ABI Prism 7900HT Sequence Detection Systems, Applied Biosystems); mRNA was quantified relative to 36B4 controls using the ΔΔCt method.

Chromatin immunoprecipitation and chromatin immunoprecipitation–microarray (ChIP-chip) analyses

Chromatin immunoprecipitation (ChIP) experiments were performed as per the Farnham protocol (25). DNA from ~1 × 107 cells was immunoprecipitated with 2 μg of anti-Myc (N262), Max, RNA polymerase II, SP1, or Miz-1 antibodies (Santa Cruz Biotechnology). PCR analysis of ChIP extracts was performed using primers that span the minimal ~500-bp Tsp-1 promoter. All primer pairs tested exhibited equivalent detection of Myc/Max binding (Supplementary Fig. S1); therefore, primers P2/P3 were used for all assays, with control primers spanning an upstream non-Myc-binding region. Ct values were normalized to that of control primers (ΔCt = Ccontrol - Ctarget), and ΔΔCt (ΔΔCt = ΔCtarget - ΔCcontrol) method was used to calculate binding enrichment relative to input DNA.

For ChIP-chip analysis, 10 pooled unamplified Myc ChIP reactions or no-antibody control samples and input genomic DNA were labeled by random priming with aminolink-UTP (Sigma) and hybridized to the University Health Network (UHN) human 12K CpG island (CGI) arrays (26) as previously described (27). Data were normalized using a robust-spline algorithm, and log2-normalized intensity ratios were imported into Excel (Microsoft). Candidate Myc-bound loci with ≥2-fold binding enrichment (Myc versus no-antibody control) in three independent experiments were selected and mapped using the UHN CpG microarray database (28).

Gene expression analysis of primary medulloblastoma

Tsp-1 and Myc expression levels in primary medulloblastoma were determined from GeneChip Human Exon 1.0 ST Array data generated in a prior study (29).

Electromobility shift assays

Electromobility shift assays (EMSA) were performed as previously described (30) using 5 μg of nuclear extract, 2 μl of antibodies, and γ-32P-labeled probe. For competitive EMSAs, reactions were preincubated with unlabeled oligonucleotides, and DNA-protein complexes were visualized after nondenaturing PAGE and autoradiography analysis.

Promoter-reporter assays

pGL3–Tsp-1 reporter-promoter plasmids, pTSp-1.1, pTsp-0.42, and the pTsp-SP1 mutant were previously described (31) and kindly provided by Dr. S. Cohn (University of Chicago). Plasmid Tsp-SP1 III with a mutant SP1 motif at −381 was generated by QuikChange Site-Directed Mutagenesis kit (Stratagene) using pTsp-1.1 as template; Myc, Myc mutant W135E, JPO2, and ΔJPO2 expression plasmids in pcDNA3.1(+) or CMV10 vectors were reported previously (22). For reporter-luciferase assays, cells were transfected with reporter-promoter plasmid(s) and control Renilla luciferase plasmid (pRL-SV40, Promega) using Lipofectamine (Invitrogen) and assayed 48 hours later using the Dual Luciferase Assay kit (Promega); all luciferase readings were normalized to Renilla activity.

RNA interference and short hairpin RNA stable cell line

For transient knockdown of Myc, D283 cells were transfected with small interfering RNA (siRNA: AM4250, Ambion, Inc.) and Silencer negative control (AM4611, Ambion) using Lipofectamine 2000 (Invitrogen), and cells were harvested 48 hours later. For stable Myc knockdown, lentiviral pGIPZ (Open Biosystems/Thermo Scientific) constructs with human c-Myc short hairpin RNAs (shRNA1 and shRNA2) were transfected into D458 cells and selected in puromycin-containing media. Myc knockdown was confirmed by quantitative
reverse transcription-PCR (qRT-PCR) and Western blot analysis.

**Cell growth, invasion, and migration assays**

Cell growth was assessed with the Roche Colorimetric Cell Proliferation/MTT assay at regular intervals as per standard protocols, and results were verified by direct cell counts for a subset of cell lines.

To assess cell migration/invasion, 0.5 × 10⁵ to 1 × 10⁵ cells were seeded into Matrigel Invasion Chambers (BD Biosciences) prehydrated with 10% FBS-αMEM. Migrated cells were quantified by staining with 1% toluidine and direct counting of 10 random microscopic fields per membrane.

For wound-healing assays, a scratch/wound was introduced with a pipette tip in near-confluent UW228-MycER cells grown in αMEM with 0.25% FBS. Cells were switched to 2.5% FBS media with 1 μmol/L 4-hydroxytamoxifen (OHT) to induce Myc expression; the width of wound in induced and uninduced cells was measured at regular intervals over 30 hours using phase-contrast microscopy. Relative cell migration was derived from changes in wound width calculated as [(measured width/2) − (width at time 0)/(width at time 0)].

**Soft agar colony formation and orthotopic xenograft assays**

Soft agar colony assays were performed as previously published (22). For orthotopic xenograft (Animal Care Committee–approved protocol), cerebella of 5- to 6-week-old anesthetized male Crl:NU-Foxn1nu nude mice (Charles River Canada) were injected with a PBS-Matrigel (BD Biosciences) suspension containing 1.0 × 10⁵ log-phase UW228-Myc, UW426-Myc, or control GFP cells. Each test and corresponding control lines were injected into 10 individual mice; all animals were euthanized as per tumor endpoint monitoring guidelines or after 12 weeks of observation. Histopathologic analysis of whole brain and spine from all mice was performed (C.H.). All oligonucleotide/primer sequences are listed in Supplementary Table S1.

**Results**

**High Myc expression confers aggressive and metastatic phenotypes in medulloblastoma cells**

To elucidate mechanisms by which Myc confers poor metastatic medulloblastoma outcomes, we characterized the effect of high ectopic Myc expression on *in vitro* and *in vivo* characteristics of two human medulloblastoma cell lines, UW228 and UW426, with low endogenous Myc levels. As Myc amplification is associated with human large cell anaplastic medulloblastoma, an aggressive histologic variant with propensity for dissemination, we inquired if high Myc expression alters cell migratory and/or invasive phenotypes in UW228 and UW426 cells. Indeed, UW228-Myc and UW426-Myc cells exhibited significantly enhanced cell migration compared with control cells in Transwell invasion assays (Fig. 1A), and induction of Myc increased migration of UW228-MycER in wound-healing assays (Fig. 1B).

High Myc expression also had potent *in vivo* oncogenic effects on UW228 and UW426 cells. Whereas mice orthotopically injected with control cell lines exhibited no growth or limited hyperplasia at injection sites (Fig. 1D, i), 8 of 10 and 9 of 10 mice injected with UW228-Myc or UW426-Myc, respectively, had rapidly growing cerebellar tumors and significantly shortened survival (Fig. 1C, P < 0.05). Examination of UW228-Myc and UW426-Myc xenografts revealed histologic features resembling large cell anaplastic human medulloblastoma (32) with characteristic large nuclei, atypical and increased mitosis, and occasional cell molding (Fig. 1D, ii, iii, and iv). Significantly, we also detected metastatic foci in spinal cord of four of eight UW228-Myc and four of nine UW426-Myc tumor-bearing mice (Fig. 1D, v).

These data collectively show that high Myc expression in medulloblastoma cell lines confers two potent oncogenic traits, increased cell migration and invasion, and also recapitulates clinicopathologic features of aggressive Myc-amplified human medulloblastoma.

**ChIP-chip analysis identifies direct Myc target genes with functions in cell adhesion and migration in medulloblastoma**

To identify effector genes important in Myc-associated metastatic medulloblastoma phenotypes, we performed ChIP-chip analyses on UW228-Myc cells using a genomic array of 12,000 CpG island–associated clones (26). We identified 1,439 candidate Myc target genes with binding enrichment ≥2-fold in three biologically independent experiments (Supplementary Table S2). We confirmed reliability and robustness of our ChIP-chip data by characterizing a subset of known Myc target loci (28), with Myc-binding sites close to known/predicted transcriptional start sites (TSS; −5 kb to +1 kb), by conventional ChIP, and by RT-PCR analysis for Myc responsiveness in four different cell backgrounds (UW228-Myc, UW426-Myc, UW228-MycER, and HO15.19-Myc, a Myc-null rat fibroblast cell line reconstituted with Myc; ref. 33). These validation analyses are shown in Supplementary Fig. S2A to C.

To uncover Myc target genes that direct the migratory and metastatic phenotypes of the UW228-Myc and UW426-Myc cell lines and xenografts, we analyzed the Myc target gene list using the DAVID annotation tool, which classifies genes based on functional similarities (34). Consistent with studies in other systems, our analyses revealed that Myc most frequently targets metabolic and transcriptional regulatory genes. Notably, DAVID analysis revealed a subset of 30 Myc candidate targets (Supplementary Table S3) with predicted functions in cell motility/migration and adhesion. Although most of the candidate targets showed Myc responsiveness in at least one of the stable or inducible Myc cell systems, we focused on further studies of only *Tsp-1/THBS1, ING4, PVRL3,* and *PPAP2B* loci, as they showed the most consistent response to Myc levels in all four cell systems (Fig. 2A). ChIP analyses confirmed Myc enrichment at these candidate promoters corresponding to Myc responsiveness of the target.
genes in UW228-Myc, UW426-Myc, HO15.19-Myc (Fig. 2B), and UW228-MycER cells (Fig. 2C).

To assess the relative importance of Tsp-1, ING4, PVRL3, and PPAP2 in Myc-mediated metastatic phenotypes, we analyzed target gene expression relative to Myc in a panel of medulloblastoma cell lines. Notably, Tsp-1 expression showed the most robust and consistent negative correlation with Myc levels in medulloblastoma cells; specifically, medulloblastoma cell lines (D283, D341, D425, and D458) with Myc amplification or high-level copy gains had low or undetectable Tsp-1 mRNA or protein expression, whereas cell lines with low Myc levels had elevated Tsp-1 expression (Fig. 3A).

To determine if Myc and Tsp-1 expression also negatively correlates in primary tumors, we analyzed a previously generated gene expression data set of 103 primary medulloblastoma and selected cell lines for relative Myc and Tsp-1 expression.

Figure 1. High Myc expression confers invasive and metastatic medulloblastoma phenotypes. A, UW228-Myc and UW426-Myc cell lines exhibited increased cell invasion/migration relative to vector control lines in Boyden chamber assays (n = 4). Bars, SD. Bottom, Western blot shows Myc levels in UW228-Myc and UW426-Myc cells comparable with that in Myc-amplified medulloblastoma cell lines D341 and D458. β-Actin served as a loading control. B, wound-healing assays showed increased migration of UW228-MycER cells with OHT-induced Myc expression compared with uninduced control cells (n = 3, two replicas per data point). Induction of a MycER fusion protein (~105 kDa) in UW228-MycER cells and accompanying decrease in endogenous Myc due to Myc autoregulation was confirmed by Western blot with β-actin as a loading control. C, survival curves for nude mice bearing cerebellar xenografts of UW228-Myc, UW426-Myc, or corresponding GFP control cells. Log-phase cells from test or control cell lines were orthotopically injected into 10 nu/nu mice. Only mice that survived >1 wk after injections were included; mice with UW228-Myc (n = 8) or UW426-Myc (n = 9) xenografts exhibited rapid brain tumor growth and significantly shorter survival than mice injected with control GFP cells that did not develop brain tumors (P = 0.014 and 0.04, respectively).
expression (29). First, qRT-PCR analysis was used to validate microarray gene expression values in a random subset of 38 of 103 tumors and 4 medulloblastoma cell lines with known Myc genomic status. Except for two tumors, microarray data correlated with RT-PCR analysis (Supplementary Table S4). We then used validated Myc and Tsp-1 expression values in four well-characterized medulloblastoma cell lines to establish thresholds for segregating the remaining tumors into high and low Myc- or Tsp-1–expressing groups. Tsp-1 expression levels (≥600 arbitrary units), which correlated with validated high Tsp-1 mRNA and protein expression in the UW228 cells, were set to distinguish low and high Tsp-1–expressing tumors. Similarly, validated Myc expression levels in the Daoy and UW228 cell lines (which do not have Myc amplification) were used to set a relative threshold of ≥1,000 arbitrary units to define low and high Myc expression in primary tumors. Based on these criteria, we identified high Myc expression in 10% to 15% (13 of 103) of primary medulloblastoma, a proportion consistent with prior studies of primary medulloblastoma (3, 7). Importantly, these analyses showed that high Myc levels also correlated significantly with low Tsp-1 expression in primary medulloblastoma (P = 0.032; Fig. 3B; Table 1).

**Myc and metastasis-associated cofactor, JPO2, synergistically repress Tsp-1 transcription in medulloblastoma cells**

Prior studies, as well as our observations in medulloblastoma cells (Supplementary Fig. S3), indicate that optimal Tsp-1 transcription requires an ~1-kb region spanning the Tsp-1 5′ upstream and intron 1 region (~954 to +147) but is also efficiently mediated by a minimal core promoter region of ~400 to 500 bp. The CpG island clone identified in UW228-Myc ChIP-chip studies mapped to intron 1 and spanned the Tsp-1 TSS, indicating that Myc may regulate the core Tsp-1 promoter. Indeed, ChIP analysis revealed colocalization of Max with Myc at the minimal Tsp-1 promoter in UW228-Myc and ONS76 cell lines, respectively, with ectopic and endogenous Myc expression (Fig. 4A). Consistent with a direct role for Myc in transcriptional repression of Tsp-1, RNA polymerase II binding at the Tsp-1 promoter was significantly diminished in UW228-Myc compared with control cell lines (~3-fold; P < 0.005; Fig. 4B). These observations were corroborated by promoter-reporter assays in UW228 and SKNBE neuroblastoma cells, which revealed significant repression of Tsp-1 promoter activity (P < 0.05) by Myc and more modest repressive effects of W135E (Fig. 4C), a Myc mutant defective in gene regulation (35).

Myc may mediate transcriptional repression by displacement of activators that bind CG-rich motifs such as Miz-1 (16) and SP1/SP3 (36). The core Tsp-1 promoter contains three conserved GC-rich/SP1-like motifs (~118, ~165, and ~581); however, neither SP1 nor Miz-1 exhibited differential promoter binding in UW228 cells (Supplementary Fig. S4). Interestingly, JPO2, a Myc cotransforming interactor we previously implicated in metastatic medulloblastoma (22), has also been reported to mediate transcriptional regulation via GC-rich/SP1-like motifs (37). Tsp-1 expression was increased in UW228 cells with JPO2 knockdown (Fig. 5A), and ChIP assays with two different JPO2-specific antibodies revealed...
JPO2 coenrichment with Myc at the core Tsp-1 promoter (Fig. 5B). To corroborate these data, which indicate that JPO2 directly contributes to Myc-mediated Tsp-1 repression, we tested the effects of JPO2 and ΔJPO2, a mutant with defective Myc binding (22), on activity of a Tsp-1 promoter construct. As shown in Fig. 5C, Myc repression of the Tsp-1 promoter was augmented by coexpression of JPO2 and, to a lesser extent, by the ΔJPO2 mutant. These findings, which indicate that Tsp-1 repression is in part mediated by direct synergistic Myc-JPO2 interactions at the Tsp-1 promoter, prompted us to test if any of the three GC-rich/SP1 motifs in the core Tsp-1 promoter were directly bound by JPO2.

EMSAs with oligonucleotide probes I, II, and III corresponding to each of the three GC-rich motifs in the Tsp-1 promoter revealed only altered mobility of probe III with anti-JPO2 antibodies but not control rabbit sera (Fig. 5D, lanes 1–9). To confirm JPO2-binding specificity, cold oligonucleotides with complete or partial substitution of the SP1-III GGGCGG motif (probes III-2 and III-3, respectively) were used to compete with radiolabeled wild-type probe III. In contrast to preincubation with cold wild-type probe, JPO2-DNA complexes were not altered by unlabeled mutant probe III-2 but was diminished in the presence of unlabeled probe III-3 with a partially preserved SP1 core motif in UW228 cells with ectopic (Fig. 5D, lanes 10–15) and endogenous JPO2 expression (Supplementary Fig. S5). These data confirm that JPO2 directly binds the Tsp-1 promoter, and indicate that transcriptional repression of Tsp-1 by Myc is
facilitated by recruitment of Myc by JPO2 and direct Myc-JPO2 interactions at the basal Tsp-1 promoter in medulloblastoma cells.

Tsp-1 suppresses Myc-mediated malignant phenotypes in medulloblastoma cells

Our observations that Myc and JPO2 independently and synergistically repress Tsp-1 transcription suggest that Tsp-1 downregulation may be important for Myc-mediated aggressive medulloblastoma phenotypes. We tested if stable Tsp-1 expression altered oncogenic phenotypes of UW228-Myc, UW426-Myc, and D458, a medulloblastoma cell line with Myc amplification and low Tsp-1 expression. Remarkably, although Tsp-1 expression did not alter cell proliferation (Fig. 6A), ectopic Tsp-1 expression in all three cell lines resulted in diminished soft agar colony growth (Fig. 6B) and striking reduction in cell invasion and migration (Fig. 6C). Similarly, we observed that stable shRNA or transient siRNA-mediated Myc knockdown was accompanied by increased Tsp-1 mRNA and protein expression and diminished cell transformation, migration, and invasion in both D458 and D283 cells, which have Myc gene amplification (Fig. 6D and E; Supplementary Fig. S6). Notably, we observed that expression of Myc-W135E and Myc-ΔMBII mutants, which, respectively, have a point mutation and a deletion in an NH2-terminal transcriptional regulatory domain, had significantly lesser effects on Tsp-1 expression compared with wild-type Myc in UW228 and UW426 cells (Supplementary Fig. S7). In aggregate, our findings indicate that Tsp-1 expression is directly and tightly regulated by Myc in medulloblastoma, and suggest downregulation of Tsp-1 as an essential step in Myc-mediated cell migration and invasion in metastatic medulloblastoma.

Discussion

Myc amplification is associated with poor medulloblastoma survival; however, biological mechanisms underlying this correlation remain to be elucidated. Here, we determined using orthotopic xenograft models that Myc expression can induce aggressive, metastatic medulloblastoma in part by direct regulation of genes with functions in cell migration and adhesion. We identify Tsp-1, a potent tumor suppressor, as a transcriptional target of Myc and metastasis-associated cofactor JPO2 in medulloblastoma cells. Importantly, we show that Tsp-1 expression robustly and specifically abrogates Myc-induced medulloblastoma cell migration and invasion. These data suggest that silencing of Tsp-1 represents a critical step in Myc-mediated cell migration and invasion in metastatic medulloblastoma.

High Myc orthotopic xenograft models

Our orthotopic xenograft models indicate that Myc expression alone can recapitulate aggressive histology of primary Myc-amplified human medulloblastoma. In addition to these findings, which corroborate prior observations in

| Table 1. Myc and Tsp-1 expression in primary medulloblastoma |
|-----------------|-----------------|---------|
| c-Myc levels    | Tsp-1 levels    | Total   |
|                 | High | Low |       |
| High           | 0    | 13  | 13     |
| Low            | 22   | 66  | 88     |
| Total          | 22   | 79  | 101    |

NOTE: $P = 0.032$, Fisher’s exact test.

Figure 3. Expression of Myc and Tsp-1 in medulloblastoma cell lines and primary tumors. A, RT-PCR and Western blot (WB) analysis of medulloblastoma cell lines with (D283, D341, D425, and D458) and without (UW228, UW426, ONS76, and Daoy) Myc amplification shows negative correlation of Myc and Tsp-1 mRNA and protein expression; 36B4 and tubulin represent loading controls. B, microarray data of 101 primary medulloblastoma (Supplementary Table S4) were analyzed for relative Tsp-1 and Myc gene expression levels. Plot shows individual tumors identified with high or low Tsp-1 and/or Myc expression levels based on thresholds (dotted lines) defined by PCR-validated Tsp-1 and Myc mRNA and protein levels in medulloblastoma cell lines (stars) as described in text; solid dots indicate two tumors for which gene expression values did not validate with qRT-PCR. Significance of correlation between Myc and Tsp-1 expression in primary medulloblastoma is summarized in Table 1.
that Myc expression directly enhances cell migration and invasion and confers highly invasive and metastatic medulloblastoma phenotypes. Notably, we observed leptomeningeal invasion and spinal metastasis in Myc orthotopic xenograft tumors, which mimic the characteristic pattern of metastasis seen in primary human medulloblastoma. Similar to human medulloblastoma (38), we observed a high but not complete correlation of metastatic disease with Myc-associated anaplastic tumors. An unexpected but interesting observation in our study was the invasion and infiltration of Myc-induced medulloblastoma xenografts into normal brain parenchyma. Unlike malignant brain tumors that are characteristically highly infiltrative and recur locally, the significance of tumor infiltration and invasion into normal tissues has not been specifically studied in medulloblastoma. However, as medulloblastoma predominantly recurs locally with or without metastasis (39), our observations suggest that invasive tumor biology may also contribute significantly to treatment failures in medulloblastoma.

Myc target genes

Prior studies (40, 41) estimate that ∼15% of genomic loci are regulated by Myc. The significantly greater proportion of candidate Myc target genes identified in our study (∼25% of genomic loci represented on the CpG arrays) likely reflects the preferential binding of Myc to CpG-rich genomic regions and more global array platforms used in other studies. Similar to prior studies of Myc and other transcription factors (42), our data indicate that Myc also associates with many genomic sites outside of promoters. The significance and characteristics of these findings remain to be determined and may be relevant to Myc function in global gene regulation (43).

Interestingly, although proliferation of UW228 and UW426 cells was increased with Myc expression, function enrichment analysis of Myc target genes revealed few cell proliferation or cell cycle loci and suggested that proliferative effects of Myc overexpression in medulloblastoma may largely result from indirect mechanisms. Of note, in addition to cell mobility and adhesion genes, loci with functions in cellular differentiation were also significantly enriched among Myc target genes in our study. Although the significance of cellular differentiation in medulloblastoma remains to be defined, our findings suggest that high Myc expression may impose more primitive medulloblastoma phenotypes with greater cell migratory and invasive capacity.

Tsp-1 and other Myc target genes with functions in cell migration

Up to 7% of candidate Myc targets identified in our study have functions in cell adhesion/motility. Novel targets of interest include ING4, a known tumor suppressor (44), and PVRL3 (45) and PPAP2B (46), which have no known oncogenic functions but are linked to regulation of the cadherin/catenin/WNT pathway.

We identified Tsp-1 as one of the most robust Myc-regulated target gene in medulloblastoma. Although Tsp-1 has not been implicated in medulloblastoma biology to date, substantial
Figure 5. Myc and cofactor JPO2 synergize to mediate Tsp-1 gene repression. A, RT-PCR analysis of JPO2 and Tsp-1 expression in UW228 with stable expression or RNA interference–mediated knockdown of JPO2 indicates negative correlation of Tsp-1 and JPO2 expression; 36B4 served as a loading control. B, a representative (n = 4) ChIP assay of the core Tsp-1 promoter performed in UW228-JPO2 cells using anti-Myc and COOH- and NH2-terminal anti-JPO2 antibodies; negative control samples processed without specific antibodies and input DNA dilution are indicated. C, luciferase assays were performed in UW228 cells transfected with a Tsp-1 promoter construct (−954 to +147 bp) and plasmids encoding Myc and/or wild-type JPO2 or mutant ΔJPO2 defective for Myc binding (n = 3, two replicas per experiment; ref. 22). Bars, SD. Structure and Myc-binding activity of JPO2 and ΔJPO2 protein are diagrammed. D, EMSA of the core Tsp-1 promoter performed with 32P-oligonucleotides containing predicted JPO2-binding motifs at positions −118 (probe I, lanes 1–3), −165 (probes II, lanes 4–6), and −581 (probe III, lanes 7–9) and nuclear lysates from UW228-JPO2 cells and anti-C-JPO2 (COOH-terminal) antibody or control revealed that only probe III exhibited specific band shifting with anti-JPO2 antibodies. ** and *, location of probe III–JPO2 complex bands after treatment with anti-JPO2 antibodies or control sera, respectively. 32P-probe III–JPO2 complex (**) was significantly diminished by pre-incubation with wild type probe III-1 and probe III-3 (lanes 13, 15) but not with probe III-2 (lane 14). Arrows indicate a non-specific band seen variably in nuclear lysates.
Figure 6. Tsp-1 suppresses Myc-mediated malignant phenotypes in medulloblastoma cells. A, MTT assays of UW228-Myc, UW426-Myc, and D458 cells with stable Tsp-1 expression and control cell lines (n = 2, six replicas per data point). Western blot confirming high Tsp-1 expression in stable cell lines relative to corresponding controls is shown with tubulin loading control. B, stable Tsp-1 expression inhibited soft agar colony formation by 15% to 17% in UW228-Myc and UW426-Myc cells and by 50% in Myc-amplified D458 cells relative to vector controls (n = 3, three replicas per data point). Bars, SD. C, Boyden chamber assays show that ectopic Tsp-1 expression diminished cell migration/invasion by 94%, 82%, and 50% in UW228-Myc, UW426-Myc, and D458 cells, respectively, relative to controls (n = 3, two replicas per experiment). Bars, SD. D, stable Myc knockdown with two different shRNAs significantly diminished D458 cell migration/invasion in Boyden chamber assays by 63% and 86%, respectively, relative to vector control cells (n = 2, three replicas per experiment). Bars, SD. Western blot analysis shows relative Myc and Tsp-1 levels in control and D458-shRNA Myc cells with a tubulin loading control (corresponding densitometry analysis and qRT-PCR assays are shown in Supplementary Fig. S6A). E, cell migration/invasion of D283 cells was diminished by up to 45% after 72 h of treatment with 40 and 80 nmol/L Myc-specific siRNA; scrambled siRNA-treated cells served as controls in Boyden chamber assays (n = 2, three replicas per experiment). Bars, SD. Changes in Myc and Tsp-1 protein and mRNA levels were confirmed by Western blot (right) and qRT-PCR (Supplementary Fig. S6B).
evidence links Tsp-1 to Myc-mediated oncogenesis. In particular, Tsp-1 has been shown to be an important component of the Myc-Ras transformation cascade (47) that promotes in vivo tumorigenesis via modulation of angiogenesis (48). The role and significance of Tsp-1 in medulloblastoma angiogenesis are not known; however, limited studies suggest that angiogenesis is not predictive of medulloblastoma biology or metastatic potential (49, 50), and hence, other cellular mechanisms may play more critical roles in medulloblastoma recurrence and metastasis. Our data, which show that one of the major cellular effects of the Myc–Tsp-1 regulatory axis is on medulloblastoma cell migration and invasion, suggest that Tsp-1 repression by oncogenic Myc may be a critical determinant of metastatic phenotypes in primary medulloblastoma.

**Myc-mediated Tsp-1 repression in medulloblastoma**

Our observation that Myc represses Tsp-1 transcription is consistent with prior studies in rat fibroblasts indicating direct and indirect mechanisms for Myc-mediated Tsp-1 regulation (51). The magnitude of change in Tsp-1 mRNA levels (50–75% of controls) in Myc-transformed medulloblastoma cells (Supplementary Fig. S8) closely parallels the extent of Myc-induced decrease in Tsp-1 promoter activity observed in our studies and indicates that transcriptional repression represents a significant mechanism by which Myc regulates Tsp-1 expression in medulloblastoma cells. Studies in mouse colonocytes reveal that Myc may also regulate Tsp-1 expression via activation of the **miR-17-92** locus, which targets Tsp-1 mRNA stability (52). Although the contribution of indirect mechanisms to Myc regulation of Tsp-1 in medulloblastoma cells remains to be studied, it is interesting to note that miR-17-92 is upregulated in a proportion of medulloblastoma cells with Myc amplification (53); thus, miR-17-92 may also contribute to Myc-mediated regulation of Tsp-1 in medulloblastoma.

As reported in rat fibroblast (54), we did not detect Myc binding to loose “E-box–like” elements at position −273/−268 of the core Tsp-1 promoter (data not shown). Although the most proximal SP1 site in the core Tsp-1 promoter is important for basal murine and human Tsp-1 expression in other cell types (31), our data indicate the distal SP1-III site, which binds JPO2, is most critical for Tsp-1 activation in medulloblastoma cells (Supplementary Fig. S3). The identity of transcriptional activators, which bind this important GC-rich site and represent potential protein targets of Myc and/or JPO2-mediated Tsp-1 repression, remains to be defined. Our data, which implicate JPO2 in Myc-mediated Tsp-1 repression, are consistent with preliminary studies indicating that JPO2 also confers migratory medulloblastoma phenotypes. Taken together with our prior observations that JPO2 expression correlates with metastatic medulloblastoma (22) and reported positive feedback regulation of Myc and JPO2 (37, 40), our data suggest that the Myc-regulatory network acts at multiple levels to tightly regulate Tsp-1 expression in medulloblastoma cells, and underscore a critical role for Tsp-1 in medulloblastoma development and progression.

The lack of knowledge about determinants of medulloblastoma progression limits further therapeutic advances in childhood medulloblastoma. By developing and interrogating xenograft models that recapitulate the human disease, our study has provided one of the first mechanistic insights into how Myc directs aggressive medulloblastoma phenotypes. In addition to providing a biological rationale for considering available Tsp-1 agonists (55) in medulloblastoma therapy, data generated from this study serve as an important basis for developing future preclinical models of metastatic medulloblastoma.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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