OTX2 Is Critical for the Maintenance and Progression of Shh-Independent Medulloblastomas

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Abstract

OTX2 is a developmentally regulated transcription factor involved in early morphogenesis of the central nervous system. This gene is amplified and overexpressed in medulloblastoma cell lines, but the nature and extent of its genetic alterations in primary tumors have not been evaluated. Analysis of a large cohort of primary medulloblastomas revealed frequent focal copy number gain of a region minimally containing OTX2 as a single gene. OTX2 copy number gain was restricted to tumor subtypes that did not express a molecular signature of Wnt or Shh pathway activation. FISH analysis revealed copy number gain in a subset of cells within medulloblastoma samples, suggesting a late event in tumor progression. Gain of OTX2 copy number was associated with the presence of anaplastic histologic features and shorter survival in medulloblastoma patients. In support of a functional role, ectopic OTX2 expression enhanced proliferation and tumorigenicity of immortalized primary cells, whereas OTX2 knockdown in medulloblastoma cells prolonged the survival of animals bearing xenograft tumors. Mechanistic investigations revealed upregulation of MYC as a potential mechanism whereby OTX2 promotes tumor progression. Our findings define OTX2 as an important oncogenic driver in medulloblastoma.

Introduction

Medulloblastoma is the most common malignant brain tumor in children, with a peak incidence at 7 years of age (1–3). In recent years, medulloblastoma has been redefined by the development of treatment strategies, including surgical resection, radiation, and chemotherapy. Still, an unacceptably large percentage of children die of this disease, with 5-year survival rates reaching only 50% to 60% (4–6). Moreover, long-term survivors frequently suffer life-long sequelae from aggressive treatment regimens (7–9). More refined tumor grading systems are therefore urgently needed to optimize efficacy and minimize treatment-related toxicity for each patient.

Histologically, medulloblastomas exhibit substantial heterogeneity both among and within tumors, and histologic characteristics do not uniformly correlate with clinical prognosis or treatment response. However, molecular analysis of medulloblastomas has shown that incorporating gene expression and genetic alteration data along with clinical and histopathologic data into a patient’s profile may better stratify patients into particular treatment and prognostic groups (5, 10–13).

Recently, we and others identified OTX2 amplification and overexpression in medulloblastoma cell lines and primary tumors (14–17). Gene expression analyses revealed that OTX2 transcripts were present at high levels in 14 of 15 (93%) medulloblastomas with anaplastic histopathologic features. Additionally, we showed that knockdown of OTX2 expression by small interfering RNAs (siRNA) inhibited medulloblastoma cell growth in vitro (15).

OTX2 is a member of a well-conserved family of bicoid-like homeodomain (HD)–containing transcription factors that play important roles in embryo patterning, brain regionalization, and lineage specification (18). OTX2 is expressed in the prospective cerebellum (the midbrain/hindbrain boundary) of the early embryo as well as in the rapidly expanding population of cerebellar granule cell precursors (19, 20). OTX2-deficient mice fail to develop the mesencephalon and prosencephalon, and heterozygotes exhibit a lethal “headless” phenotype of variable penetrance (21, 22). Although the expression pattern and function of OTX2 in brain morphogenesis have been well studied in progressive developmental stages of rodent models (20, 23), there is little understanding of how OTX2 relates to the clinical and
histopathologic features of medulloblastoma or to the molecular pathways of medulloblastoma pathogenesis.

To further our understanding of the genetic mechanism of OTX2 activation and its clinical implications in medulloblastomas, we examined its copy number and expression status in a large cohort of tumors (201 and 103 primary medulloblastomas, respectively). Here, we identify recurrent OTX2 gain (∼74%) and, even more commonly, OTX2 overexpression (∼21%) in primary medulloblastomas. OTX2 focal gains were limited to tumors not expressing signatures of Wnt or Shh pathway activation. Fluorescence in situ hybridization (FISH) analysis showed that OTX2 copy number gain was present in a subset of medulloblastoma cells and was associated with a more aggressive tumor phenotype and adverse clinical outcome. To understand its oncogenic functions, we overexpressed OTX2 in immortalized primary cells and showed that OTX2 could promote cell proliferation and tumorigenicity. Furthermore, we determined that stable OTX2 knockdown prolonged survival in a medulloblastoma xenograft model. Finally, we showed that OTX2 transcriptionally regulates the medulloblastoma oncogene MYC. These results suggest that OTX2 plays a role in progression and maintenance of a large subset of medulloblastoma tumors and thus represents a promising therapeutic target.

Materials and Methods

Patients. Medulloblastomas were obtained from the Duke University Medical Center Brain Tumor Biorepository and the Hospital for Sick Children, University of Toronto, according to Internal Review Board protocol. This biorepository contained specimens obtained from Duke and the (now defunct) Pediatric Oncology Group that had been originally studied for MYC amplification (24).

Pathology. Classic, desmoplastic/nodular, and anaplastic/large cell medulloblastomas used in FISH analysis were examined by a neuropathologist (R.E.M.) and classified using the WHO guidelines (1). The classic, or diffuse, pattern of medulloblastoma presents a histologic appearance of densely packed cells with round-to-oval or carrot-shaped hyperchromatic nuclei surrounded by scanty cytoplasm growing in sheets. Neuroblastic, or Homer-Wright, rosettes are most commonly encountered in this group (25).

The anaplastic variant is composed of cells with elongated hyperchromatic nuclei that are densely crowded and exhibit characteristic nuclear wrapping against adjacent tumor cells, abundant individual cell necrosis frequently associated with geographic regions of necrosis, and numerous mitotic figures (26). Moderate anaplasia lacks the degree and extent of necrosis seen in severely anaplastic tumors and is more commonly recognized by a "starry sky" pattern of individual cell necrosis interrupting the sheets of tumor cells.

Single-nucleotide polymorphism array and microarray analysis. Hybridization, detection, and analysis of Affymetrix single-nucleotide polymorphism (SNP) and exon arrays were carried out as previously described (27, 28).

Fluorescence in situ hybridization. FISH was performed as described previously (29). Bacterial artificial chromosome clones RP11-1085N6 (56.159277-56.402022 Mb, spanning the OTX2 gene) and CTD-2505F22 (103.186989-103.41534 Mb, located telomeric to OTX2 on chromosome 14q) were obtained (Invitrogen) and used to generate probes for the OTX2 gene and an internal control, respectively. One hundred cells were counted in each of 10 classic medulloblastomas, 11 desmoplastic/nodular medulloblastomas, 10 medulloblastomas with moderate anaplastic features, and 11 medulloblastomas with severe anaplastic features. OTX2 copy number was determined by the average ratio of green to red probes per cell, and copy number gain was designated as tumors with a ratio >1.2.

Statistical analysis. The significance level for all tests was set a priori at 0.05 by using a 95% confidence interval (95% CI). All statistical and survival analyses were performed with SAS E-guide software (SAS Institute).

Plasmid constructs. A full-length cDNA of human OTX2 was derived from pCMV6-XL5-OTX2 (OriGene) and subcloned into the pEGFP-N1 vector.

Cell culture, proliferation, and apoptosis assays. Medulloblastoma and rat kidney epithelium (RK3E) cell lines were maintained as described previously (15, 30). Cell line D425MED was derived from a patient with a primary medulloblastoma, whereas D458MED was established independently from cerebrospinal fluid samples of the same patient at a later date. MIT and colony proliferation assays were performed as described (15, 30).

Establishing an RK3E line stably expressing OTX2. RK3E cells were transfected with pEGFP-N1 or pEGFP-OTX2. The transfected cells were cultured in DMEM containing 500 μg/mL G418 (Invitrogen) for 3 to 4 wk. Single-cell dilution was used to select monoclonal cell lines that were resistant to G418 and expressing OTX2.

Tumorigenicity in athymic nude mice. Groups of seven or eight mice per cell line were used to investigate the tumorigenicity of RK3E cells stably transfected with pEGFP-N1 or pEGFP-OTX2, respectively. We implanted 106 cells stereotactically into the cerebral hemisphere of BALB/c athymic nu/nu mice. Brains of euthanized mice were collected, fixed in formalin, paraffin embedded, and sectioned. The presence of intracranial tumors, indicated by parenchymal invasion, was assessed microscopically in multiple brain sections. The tumor proliferation activity was examined by Ki67 immunoreactivity using a Ki67 monoclonal antibody (Lab Vision). OTX2 immunoreactivity was detected with biotinylated anti-human OTX2 antibody (BAF1979; R&D Systems) followed by horseradish peroxidase (HRP)–conjugated secondary antibody.

Immunoblotting. OTX2 was detected with biotinylated anti-human OTX2 antibody (BAF1979) and HRP-rabbit anti-goat IgG secondary antibody (Zymed), MYC and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were detected with 9E10 (Santa Cruz Biotechnology) and FL-335 (Santa Cruz Biotechnology), respectively, and enhanced chemiluminescence anti-rabbit IgG secondary antibody (Amersham Biosciences).

siRNA and short hairpin RNA of OTX2 and MYC. Medulloblastoma cells were plated at 2 × 105 per well into a six-well
plate and transfected with siRNA or short hairpin RNA (shRNA) using Lipofectamine 2000 (Invitrogen). siRNA and shRNA sequences used are described in Supplementary Materials and Methods.

Animal survival studies. Approximately $10^7$ D425MED cells were transfected with scrambled shRNA or OTX2 shRNA, suspended in 10 μL PBS, and stereotactically introduced into the right cerebral hemisphere of a BALB/c Balb/Ka Jcl female mouse (Japan SLC) under halothane anesthesia. The animals were monitored for survival and systemic signs of toxicity. The study was approved by the Institutional Animal Care and Use Committee of the National Institutes of Health (NIH).
Athymic nu/nu mouse. Mice were euthanized at the first sign of neurologic dysfunction. The date when each mouse became symptomatic was recorded to calculate the time to event. Standard Kaplan-Meier estimates were generated for each time to event, and groups were compared with log-rank tests.

Luciferase assays. For luciferase assays, we obtained the pBV-del-3-Luc plasmid, composed of a 1.1-kb genomic fragment encompassing the MYC promoter inserted upstream of a minimal promoter and the firefly luciferase reporter gene in the pBV-Luc plasmid (Supplementary Fig. S3B; ref. 31). Medulloblastoma cells were seeded at 1 × 10^5 per well in 48-well plates for 24 h, and then each well was cotransfected with 0.8 μg of firefly luciferase reporter plasmid pBV-del-3-Luc, the internal control plasmid pRL-CMV, and 0.8 μg of pEGFP-OTX2 or the control vector pEGFP-N1. Dual-Luciferase Reporter assays were performed according to the manufacturer’s protocol (Promega).

Chromatin immunoprecipitation. Chromatin immunoprecipitation (ChIP) was performed using a ChIP Assay kit (Upstate Cell Signaling Solutions) according to the manufacturer’s protocol. Immunoprecipitation was performed overnight at 4°C with biotinylated anti-human OTX2 antibody (R&D Systems). Immunoaffinity-enriched and control DNA fragments were then purified for DNA PCR amplification.

**Results**

Recurrent OTX2 focal gains and overexpression in medulloblastomas. To determine the frequency of OTX2 genetic alterations in a large cohort of primary tumors, we used high-density SNP arrays to perform a genome-wide search for genetic changes in 11 medulloblastoma cell lines and 201 primary medulloblastomas (27). The most frequent target of focal gain among the 212 samples was a region mapping to chromosome 14q23, detected in ∼10% (21 of 212) of the medulloblastomas (Fig. 1A). This locus exhibited either focal (i.e., <1 Mb) or large (i.e., 14q gain) genomic gain in a total of ∼21% (9.9% and 11.3%, respectively) of cases profiled. Examination of the minimal common region gained in these samples using the University of California Santa Cruz Genome Browser revealed that the single gene OTX2 was completely located within this minimal region of chromosomal gain (Fig. 1A). OTX2 gain of copy number indicated by SNP arrays was verified by real-time PCR on genomic DNA of selected medulloblastoma samples (Supplementary Fig. S1). The highly specific gain of OTX2 copy number in a large...
proportion of medulloblastomas implies that OTX2 gain is a driver event that confers a selective advantage during clonal expansion of this neoplasm.

In addition to harboring focal gains and activating mutations, oncogenes are commonly overexpressed to activate tumorigenic pathways. To determine the prevalence of OTX2 mRNA overexpression, we used Affymetrix exon arrays to measure OTX2 mRNA level in 9 normal fetal cerebella, 5 normal adult cerebella, and 103 primary medulloblastomas. We found that fetal cerebellum expressed OTX2 at a modestly higher level than adult cerebellum; however, among the 103 medulloblastomas, 76 tumors (74%) expressed OTX2 at significantly (i.e., >2-fold) higher levels than fetal or adult cerebellum (P < 0.001, Student’s t test; Fig. 1B and C).

To understand OTX2 amplification at the cellular level, we performed dual-color FISH to validate OTX2 amplification in three medulloblastoma cell lines previously identified to bear OTX2 amplifications. We noted OTX2 genomic amplification as double-minute chromosomes in D425MED, D458MED, and D487MED cell lines (Supplementary Fig. S2, a–c). Moreover, increased OTX2 copy number was also observed in the primary tumors from which the medulloblastoma cell lines D425MED and D487MED were derived (Supplementary Fig. S2, d and e).

Medulloblastomas can be classified into distinct molecular subgroups based on gene expression signatures (28, 32, 33). Our clustering analysis of a large cohort (n = 103) of tumors based on Affymetrix exon arrays previously revealed four distinct medulloblastoma subtypes: tumors expressing a molecular signature of Wnt pathway activation, those exhibiting a signature of Shh pathway activation, and those bearing two additional distinct molecular signatures designated group C and D tumors (28). Group C and D tumors as well as those with Wnt pathway activation are typically of the classic histopathologic subtype, whereas tumors with a Shh molecular signature are often of the nodular/desmoplastic histopathologic subtype (32, 33). Examination of OTX2 focal gains among medulloblastoma subtypes reveals that this event is limited to group C and D tumors (Fig. 2A). Additionally, OTX2 is differentially expressed in the molecular subgroups of medulloblastoma (P < 0.001, one-way ANOVA), with the most aberrant expression observed in group C and D tumors as well as tumors with a Wnt pathway signature (Fig. 2B).

OTX gain of copy number associates with anaplastic features and a worse patient outcome. To understand the clinical implication of OTX2 activation in medulloblastomas, we collected medulloblastoma samples with linked clinical

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Figure 2. OTX2 gain and overexpression are specific to medulloblastoma subgroups. A subset of the primary medulloblastomas (n = 81) analyzed in Fig. 1 was classified into four molecular subgroups based on gene expression signatures obtained from exon arrays. A, focal gain of OTX2 (red) is limited to group C and D medulloblastomas. The minimal region of gain (∼10.5 kb) is boxed. B, OTX2 mRNA overexpression is highest in group C and D tumors, as well as those expressing a signature of Wnt pathway activation.
data and studied OTX2 genetic status in these cases using FISH. A total of 39 patients with complete clinical data were available for survival analysis based on the FISH data, including 20 with tumors of classic or nodular histology and 19 with tumors of moderate to severe anaplasia. Classic and desmoplastic/nodular sections consistently showed no evidence of increased OTX2 copy number (0 of 20). However, a significant number of tumors with moderate and severe anaplastic characteristics displayed increased OTX2 copy number (12 of 19; \( P < 0.05 \); Fig. 3A). Only a subpopulation of cells in those samples revealed increased copy number, with OTX2 copy number ranging from 3 to 24 per individual cell (mean, 2.3 ± 1.7). Among the 39 patients, 12 patients who had gain of OTX2 copy number in their tumors had a shorter overall survival compared with those with diploid OTX2 copy number (\( P = 0.005 \); Fig. 3B). The 5-year survival estimate is 46% (95% CI, 24–87%) for the group with increased OTX2 copy numbers and was nearly doubled (86%; 95% CI, 74–99%) for the group with normal OTX2 copy numbers. Age stratification of this sample group revealed that survival of these patients mimics that of medulloblastoma patients in general, with age <3 years indicating poor prognosis (\( P = 0.001 \); Fig. 3C).

**OTX2 promotes cell proliferation and tumorigenicity and is essential for medulloblastoma cell survival.** To examine the transforming potential of OTX2, we transfected the pEGFP OTX2 plasmid into nonneoplastic RK3E cells, an adenovirus E1A-immortalized epithelial line that has been widely used for neoplastic transformation studies (30). This assay reveals oncogenic activity independent of cell origin effects, which is suitable considering that the cell origin of medulloblastomas harboring OTX2 focal gains (group C and D tumors) is unknown. OTX2 protein expression was detected by Western blotting (Fig. 4A). Overexpression of OTX2 promoted a significant increase in colony formation (\( P < 0.05 \); Fig. 4A). To assess the ability of OTX2 to promote in vivo tumor growth, we injected RK3E cells expressing either pEGFP-OTX2 or pEGFP-N1 into the cerebral hemispheres of nu/nu mice. We found that whereas all OTX2-expressing clones formed tumors and displayed high mitotic indices scored by Ki67 staining (Fig. 4A), green fluorescent protein (GFP)—expressing clones formed tumors much less frequently and scored relatively lower for Ki67 staining (\( P < 0.001 \); Supplementary Table S1). To verify that the transformative ability of OTX2 relies on its transcriptional activity rather than alternative functions, we introduced a loss-of-function
mutation into the OTX2 homeodomain to abolish DNA binding (Supplementary Fig. S3A; ref. 34). We then transfected MHH-1 medulloblastoma cells with either OTX2 or its HD NAAIRS mutant [pEFGP-OTX2 (K)] and measured MTT activity. As expected, wild-type OTX2 increased proliferation of MHH-1 cells, and the effect was greatly reduced in the NAAIRS mutant (Fig. 4B).

We have previously shown that OTX2 knockdown reduces viability of medulloblastoma cell lines (15). To determine if OTX2 expression is required for tumor maintenance in vivo as well as in vitro, we carried out RNA interference experiments in medulloblastoma xenografts. First, we used OTX2 siRNA or shRNA to confirm that OTX2 knockdown inhibited cell growth or survival in an expanded cohort of OTX2-expressing medulloblastoma cell lines (Supplementary Fig. S4). We then applied the pSUPER vector system to stably suppress endogenous OTX2 expression in D425MED cells (Fig. 4C) and examined whether downregulation of OTX2 expression affected survival in nude mice bearing intracranial xenografts. Although tumors were detected from both the OTX2 knockdown group and the control group, reducing OTX2 expression significantly increased the survival time of the animals from 22.6 ± 4.7 days to 30.8 ± 5.9 days (P = 0.0061; Fig. 4C).

**MYC expression is regulated by OTX2.** Amplification and overexpression of the oncogene MYC are also common in medulloblastoma, particularly in those exhibiting anaplastic histopathologic features (35). Therefore, we performed biochemical studies to investigate the relationship between OTX2 and MYC.

We first examined the correlation between OTX2 and MYC expression by Western blot in medulloblastoma cell lines that do not have OTX2 or MYC amplification. We observed

![Figure 4. OTX2 promotes tumorigenesis and is required for tumor maintenance in vivo. A. RK3E cells were transfected with pEGFP-N1 or pEFGP-OTX2 and colony formation potential was determined. Tumors derived from intracranial xenografts of stably transfected RK3E cells were stained for Ki67 and OTX2, and representative samples are pictured. Green arrows identify intratumor blood vessels. IHC, immunohistochemical. B. MHH-1 cells were transfected with pEGFP-N1, pEGFP-OTX2, or pEGFP-OTX2 (K). After three doubling times, proliferation was determined with an MTT assay. C. D425MED cells derived from OTX2 shRNA knockdown or scrambled shRNA were injected into the right cerebral hemisphere of mice. Representative H&E-stained tumor sections from each group are pictured. Mice were euthanized at the first sign of neurologic dysfunction and survival time was recorded.](https://www.aacrjournals.org/cancerres/article-pdf/70/1/187/20367295/can-09-2331.pdf)
that detectable MYC expression level was limited to cell lines expressing OTX2 (Fig. 5A). Using serial analysis of gene expression data from the Cancer Genome Anatomy Project, we found that the presence of OTX2 mRNA correlated with the presence of MYC mRNA in medulloblastomas (P = 0.0140; Supplementary Fig. S5). To determine whether OTX2 is required for MYC expression in medulloblastoma, we applied OTX2-specific siRNA to knock down OTX2 expression in medulloblastoma cell lines and found that MYC expression was also downregulated by the OTX2 siRNA (Fig. 5B). The complementary experiment in which MYC is knocked down by siRNA resulted in cell growth inhibition (Fig. 5C) but not downregulation of OTX2 (Fig. 5D). We then used a luciferase assay to show that OTX2 upregulates MYC via cis-acting elements in its promoter (Fig. 6A), which contains multiple putative OTX2 binding sites (Supplementary Fig. S5B). Finally, ChIP of fragments enriched with an anti-OTX2 antibody in cell lines expressing both MYC and OTX2 revealed direct binding of endogenous OTX2 to the MYC promoter in the context of native chromatin (Fig. 6B).

Discussion

Medulloblastoma patients have widely disparate and often unpredictable clinical outcomes. It is therefore important to develop better predictive molecular diagnostic tools that could be used in clinical practice to better define prognosis and guide therapy. In the current report, the investigation of OTX2 genetic status and its pathogenic roles in medulloblastoma oncogenesis have revealed that OTX2 genetic alteration is associated with tumor progression and poor survival and thus may have diagnostic value. Furthermore, we have shown a requirement for OTX2 in medulloblastoma viability, potentially revealing a novel therapeutic target in this tumor. We have also identified MYC as a downstream target gene of OTX2. This study advances our knowledge of the pathogenesis of medulloblastoma and has great potential to lead to significant contributions to the therapeutic intervention of this frequently lethal cancer.

The specific and recurrent focal gain of OTX2 represents a driver event in medulloblastoma tumorigenesis. OTX2 copy number gain is limited to tumors not bearing a molecular signature of Shh or Wnt pathway activation and thus represents an oncogenic event in tumor subtypes whose molecular progressions are currently unclear. The group C and D tumors that distinctly harbor OTX2 copy number gains are typically of the classic histopathologic subtype (32, 33). Considering that OTX2 gain is also associated with the presence of anaplastic features, it would be expected that there would be substantial overlap between group C or D molecular signatures and the presence of anaplasia. It has been described previously that anaplastic features can be present in variable degrees of severity and extent among the medulloblastoma histopathologic subtypes (36).

Using FISH, we found that OTX2 copy number varies widely among tumor cells, suggesting that OTX2 gain of copy number is a late event that is selected for within the tumor as a step of tumor progression. This model is consistent with the association of OTX2 copy number gain with worse patient survival as well as the presence of anaplastic features, which has been proposed to result from the progression of less aggressive tumors (37).

It remains a challenge to identify the patients with unfavorable prognoses who may benefit from the most aggressive treatment. Patient stratification is particularly important to medulloblastoma due to the deleterious effects of aggressive treatment in young patients. The Kaplan-Meier analysis revealed a significant association between increased OTX2 copy number and worse survival. Similar to other study populations, this group of patients also showed a clear association between poor survival and age <3 years, a well-known

Figure 5. MYC is a target gene of OTX2 in medulloblastoma cell lines. A, expression of endogenous OTX2 and MYC in medulloblastoma cell lines shown by Western blotting. B, medulloblastoma cell lines were treated with OTX2 siRNA (+) or scrambled siRNA (−), and protein expression of OTX2, MYC, and GAPDH was detected by Western blotting. C, cells were treated with MYC siRNA or scrambled siRNA and, after three doubling times, assessed for viability with the MTT assay. D, medulloblastoma cell lines were treated with MYC siRNA (+) or scrambled siRNA (−), and protein expression of OTX2, MYC, and GAPDH was detected by Western blotting.

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negative predictive factor for medulloblastoma. These findings support the possible use of OTX2 genetic status as an important marker for prognosis. In addition, OTX2 status may improve stratification schemes for current treatment protocols. de Haas and colleagues (38) did not observe a similar correlation between OTX2 expression and outcome, which suggests that copy number gain is a more faithful indicator of poor prognosis than overexpression alone.

Although mutation and copy number analysis of tumor specimens has revolutionized our understanding of recurrent molecular events in cancer, it is important to confirm oncogenic activity by functional studies. Here, we have shown in vitro and in vivo that OTX2 promotes cell growth and enhances tumorigenicity and that medulloblastoma cells rely on OTX2 transcriptional function for their aggressive tumorigenic phenotype. Therefore, we have shown that the highly prevalent overexpression of OTX2 in medulloblastoma has functional significance.

Recent advances in cancer genetics and the development of mouse models have shown a relationship between developmentally associated molecular pathways and medulloblastoma pathogenesis (39). The current study supports previous work implicating OTX2 as one such developmental gene involved in tumorigenesis (16, 17). Whereas medulloblastomas arising from oncogenic Shh signaling arise from committed granule cell precursors (40, 41), the cell origins of group C and D medulloblastomas (which distinctly harbor OTX2 focal gains) are currently unknown. During normal cerebellum development, OTX2 is expressed in the prospective cerebellum as well as emergent and migrating granule cell precursors (20, 38). Mouse models have shown that OTX2 coordinates lineage differentiation in the central nervous system, promoting generation of dopaminergic neurons and, in some domains, repressing lineage commitment to granule cell and serotonergic neural lineages (42, 43). Understanding the potential of OTX2 to facilitate tumorigenesis of particular cerebellar lineages will yield considerable insight into the cell origin of group C and D medulloblastomas.

The molecular classification of medulloblastoma using gene expression signatures and select genomic features has been reported by three independent laboratories to date: Thompson and colleagues (33), Kool and colleagues (32), and ours (28). In all of these studies, very similar bioinformatics was used. In each case, class discovery was achieved by performing unsupervised hierarchical clustering (Pearson correlation) using the genes showing the greatest variance in the data set. Thompson and colleagues and Kool and colleagues each identified five molecular subgroups (based on 46 and 62 primary tumors, respectively), whereas we used 90 tumors and identified four molecular subgroups. The disparity between the five versus four subgroups pertains to what we have designated group C and group D, which are equivalent to what Kool and colleagues referred to as C, D, and E. We have since analyzed a larger cohort of our own 103 cases and again generated the same four molecular subgroups (based on 46 and 62 primary tumors, respectively), whereas we used 90 tumors and identified four molecular subgroups. The disparity between the five versus four subgroups pertains to what we have designated group C and group D, which are equivalent to what Kool and colleagues referred to as C, D, and E. We have since analyzed a larger cohort of our own 103 cases and again generated the same four molecular subgroups with extremely high confidence (≥97% reproducibility). The larger sample cohort and more robust array platform (i.e., Affymetrix exon arrays versus U133 arrays) used in the current study provides increased power and statistical confidence in class discovery. Further application of the proposed molecular clustering strategies will reveal the most faithful method of tumor classification.

This study provides important insights into the mechanisms by which medulloblastomas develop through the dysregulation of OTX2, a gene normally silenced in adult cerebellum. Furthermore, these results establish OTX2 as a potential therapeutic target, the inhibition of which could result in tumor cell growth inhibition. For the future development of targeted therapies, it will be important to perform detailed molecular studies of the oncogenic pathways driven by OTX2 as well as the pathways maintaining its expression in tumors. These studies will aid the development of molecular diagnostic and therapeutic strategies for patients whose tumors present with this molecular hallmark. Once such strategies become available, there is promise that in tumors

Figure 6. The OTX2 HD is required for OTX2-mediated MYC transcription. A, MHH-1 cells were cotransfected with the MYC promoter reporter construct (pBV-del-3-Luc) and either pEGFP-N1, pEGFP-OTX2, or pEGFP-OTX2 (K), and then the relative luciferase reporter activity was measured. B, OTX2 ChIP and PCR analyses were used to validate binding of endogenous OTX2 to the promoter region of MYC (primers were designed to span the region 375 bp directly upstream of the MYC transcription start site) in OTX2-expressing cell lines. D425MED and D384MED are OTX2-expressing medulloblastoma cell lines, whereas MHH-1 does not express OTX2. , input of total DNA from cell lysates; −, OTX2 antibody immunoprecipitation-enriched DNA; −, IgG control antibody immunoprecipitation-enriched DNA.

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with OTX2 expression, the dysregulated signaling pathway can be corrected by specifically reducing OTX2 expression or inhibiting other components of this pathway.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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**References**

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