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Somatic Cell Transfer of *c-Myc* and *Bcl-2* Induces Large-Cell Anaplastic Medulloblastomas in Mice

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Abstract

A highly aggressive subgroup of the pediatric brain tumor medulloblastoma is characterized by overexpression of the proto-oncogene *c-Myc*, which encodes a transcription factor that normally maintains neural progenitor cells in an undifferentiated, proliferating state during embryonic development. *Myc*-driven medulloblastomas typically show a large-cell anaplastic (LCA) histological pattern, in which tumor cells display large, round nuclei with prominent nucleoli. This subgroup of medulloblastoma is therapeutically challenging because it is associated with a high rate of metastatic dissemination, which is a powerful predictor of short patient survival times. Genetically engineered mouse models have revealed important insights into the pathogenesis of medulloblastoma and served as preclinical testing platforms for new therapies. Here we report a new mouse model of *Myc*-driven medulloblastoma, in which tumors arise in situ after retroviral transfer and expression of *Myc* in Nestin-expressing neural progenitor cells in the cerebella of newborn mice. Tumor induction required concomitant loss of *Tp53* or overexpression of the antiapoptotic protein *Bcl-2*. Like *Myc*-driven medulloblastomas in humans, the tumors induced in mice by *Myc+Bcl-2* and *Myc-Tp53* showed LCA cytoarchitecture and a high rate of metastatic dissemination to the spine. The fact that *Myc-Tp53* tumors arose only in *Tp53*^{-/-} mice, coupled with the inefficient germline transmission of the *Tp53*-null allele, made retroviral transfer of *Myc+Bcl-2* a more practical method for generating LCA medulloblastomas. The high rate of spinal metastasis (87% of brain tumor-bearing mice) will be an asset for testing new therapies that target the most lethal aspect of medulloblastoma.

Keywords

medulloblastoma; mouse model; *Myc*; *Bcl-2*; spinal metastasis

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Introduction

Integrated genomic analysis has shown that medulloblastomas, once considered a uniform disease entity, comprise a diverse set of tumors, in which tumor growth is driven by activation of different molecular signaling pathways—reviewed in [1]. Four distinct groups of tumors (WNT, SHH, Group 3, and Group 4) are now widely recognized, in which affected patients show different age and gender distributions, rates of metastatic dissemination, and survival times. Molecular classification of medulloblastomas is important clinically because customizing therapies to target subgroup-specific signaling pathways will hopefully improve treatment response.

Group 3 medulloblastomas present the greatest therapeutic challenges to pediatric oncologists. Metastasis, a powerful predictor of short survival times, is significantly more common in Group 3 tumors compared with other subgroups, occurring in 47% of patients at initial diagnosis [2,3]. Furthermore, Group 3 medulloblastomas are prevalent in children younger than 4 years old when the neurotoxic effects of radiation therapy are prohibitively high. A striking feature of Group 3 medulloblastomas is that they often show a large-cell anaplastic (LCA) histological pattern, in which the tumor cells have large, round nuclei with prominent nucleoli.

Genetically engineered mouse models have revealed important insights into the pathogenesis of medulloblastoma and served as preclinical testing platforms for new therapies. Thus far, most mouse models recapitulate the type of medulloblastoma, in which tumor growth is driven by Sonic Hedgehog (SHH) signaling—reviewed in [4]. SHH is a secreted protein that activates a cell signaling pathway governing key phases of brain morphogenesis—reviewed in [5]. Pertinent to medulloblastoma formation, SHH signaling stimulates proliferation and blocks differentiation of neural progenitor cells in the developing cerebellum. We previously developed an *in vivo* model of this medulloblastoma subgroup by retroviral transfer and expression of the *Shh* gene in Nestin-expressing neural progenitor cells in the cerebellum of newborn mice [6]. We also showed the practical utility of the model for testing inhibitors of both SHH and hepatocyte growth factor signaling pathways [7].

The defining molecular characteristic of Group 3 medulloblastomas is overexpression and amplification of the gene encoding the oncogenic transcription factor c-Myc (hereafter called Myc) [1]. Like SHH, Myc maintains neural progenitor cells in an undifferentiated, proliferating state during normal embryogenesis—reviewed in [8]. Two research teams have created mouse models of Group 3 medulloblastomas by simultaneously overexpressing *Myc* and suppressing expression of the tumor suppressor gene *Tp53* in two different populations of neural progenitor cells and then implanting the manipulated cells into the brains of immunocompromised mice. One model used granule neuron precursors (GNPs) from mice completely lacking the *Tp53* gene (*Tp53*^{-/-} mice) as the cells of tumor origin [9]. In the second model, *Myc* and a dominant-negative *Tp53* were expressed simultaneously in a rare population of cerebellar stem cells characterized by expression of the cell-surface protein Prominin1 and lack of neuronal and glial lineage markers [10]. In both cases, the tumors

showed the signature LCA histological pattern and gene expression profiles resembling that of human Group 3 medulloblastomas.

The objective of this study was to use our retroviral transfer method to create a completely in vivo mouse model of *Myc*-driven, SHH-independent medulloblastomas. Compared with the above-described transplantation models, our method of inducing tumors by oncogene transfer in situ has the advantage of recapitulating the sporadic genetic changes that occur naturally during tumor initiation from somatic cells and of preserving the immunocompetent microenvironment of the host. Our proposed model would also obviate the need for labor-intensive preimplantation protocols for cell sorting and in vitro gene transduction.

Materials and Methods

Genetically engineered mice

The use of mice in this study was approved by the Institutional Animal Care and Use Committee of the University of Utah. Production of the *Ntv-a* transgenic mouse line, in which expression of the *tv-a* transgene is driven by promoter/enhancer sequences of the *Nestin* gene, has been described previously [11]. Because of the breeding strategy used to introduce the transgene, *Ntv-a* mice are hybrids composed of the following genetic strains: C57BL/6, BALB/C, FVB/N, and CD1. To create the *Ntv-a/Tp53*^{+/-} strain, we crossed *Ntv-a* transgenic mice with B6.12952-*Trp53*^{tm1Tyj}/J mice (obtained from the Jackson Laboratory, Bar Harbor, ME), which are heterozygous for a null allele of the *Tp53* gene [12].

In vivo somatic cell gene transfer in transgenic mice

To induce medulloblastomas in mice, we used a version of the RCAS/*tv-a* somatic cell gene transfer system to target the expression of *Myc*, *Bcl-2*, and *Shh* in Nestin⁺ neural progenitor cells in the cerebellum. This system utilizes a replication-competent, avian leukosis virus, splice acceptor (RCAS) vector, derived from the subgroup A avian leukosis virus (ALV-A), and a transgenic mouse line (*Ntv-a*) that produces TVA (the cell surface receptor for ALV-A) under control of the *Nestin* gene promoter [13]. After TVA-mediated infection of mammalian cells with RCAS retrovirus, the newly synthesized provirus integrates into the host cell genome where the transferred gene is expressed constitutively. RCAS-transduced mammalian cells do not produce infectious virus because mRNA splicing events remove the retroviral genes necessary for virus replication.

To initiate gene transfer, we injected retrovirus packaging cells (DF-1 cells transfected with and producing recombinant RCAS retrovirus) into the lateral cerebellum from an entry point just posterior to the lambdoid suture of the skull (bilateral injections of 10⁵ cells in 1–2 µl of phosphate-buffered saline). For experiments involving simultaneous transfer of two genes, we prepared cell pellets by mixing equal numbers of both retrovirus-producing cells. For transfer of *Shh* alone, a 1:1 mixture of RCAS-SHH and RCAS-LacZα (described below) producer cells was injected. We injected mice within 72 hours after birth because the number of Nestin⁺ cells decreases progressively during the course of neuronal differentiation. The mice were sacrificed when signs of increased intracranial pressure became apparent, indicated by enlarging head circumference (a sign of hydrocephalus), head

tilt, gait ataxia, or failure to eat or drink. Asymptomatic mice were sacrificed 4 months after injection. The brains were fixed in formalin and divided into quarters by parallel incisions in the coronal plane. To identify spinal metastatic dissemination, we fixed whole spinal column preparations in formalin for 48–72 hours and then removed the spinal cord by microdissection. Brain and spinal cord specimens were embedded in paraffin and sectioned for histochemical analysis.

Retroviral vector construction

RCAS retroviral vectors for gene transfer were constructed by ligating PCR-generated cDNA molecules corresponding to the entire coding sequence of the *Myc*, *Bcl-2*, and *Shh* genes into parent vector RCASBP(A) as we described previously [6,14]. The RCAS-LacZ α vector, which encodes the nononcogenic α -peptide of *Escherichia coli* β -galactosidase, was used in mixing experiments with RCAS-SHH. To produce live virus, we transfected plasmid versions of RCAS vectors into immortalized chicken fibroblasts (DF-1 cells) and allowed them to replicate in culture.

Tp53 genotyping

Using genomic DNA extracted from mouse tail tissue as a template, PCR products spanning the wild-type *Tp53* allele (450 base pairs) and mutant *Trp53^{tm1Tyj}* allele (650 base pairs) of the mouse *Tp53* gene were synthesized in a 35-cycle reaction using a protocol provided by the Jackson Laboratory ([http://jaxmice.jax.org/protocolsdb\(#002103\)](http://jaxmice.jax.org/protocolsdb(#002103))). PCR products were separated by electrophoresis through 1.5% agarose gels and visualized by UV illumination after immersion in ethidium bromide solution.

Immunocytochemistry and microscopy

Tissue sections were cut 4 μ m thick, mounted on glass slides, deparaffinized with toluene, hydrated through a descending series of ethanol, autoclaved in a citrate-based antigen retrieval solution (Vector Laboratories, Burlingame, CA) for 5 min, and cooled to room temperature. Sections were then treated with H₂O₂ (1% v/v) for 10 min to quench endogenous peroxidase activity and washed with phosphate-buffered saline. After immersion in normal horse serum (2%), sections were incubated with primary antibody in a humid chamber at 4°C overnight. Immunoreactive staining was visualized using a biotin-free reporter enzyme staining system (ImmPRESS, Vector Laboratories), which utilizes a micropolymer of peroxidase and affinity-purified secondary antibodies. Diaminobenzidine was used as the chromogenic substrate and toluidine blue as a nuclear counterstain. We used the following antibodies (and dilutions) from the indicated commercial sources: Mab9E10 (1:50)—human-specific c-Myc epitope EQKLISEEDL (Santa Cruz Biotechnology, Santa Cruz, CA); Mab100 (1:50)—human-specific Bcl-2 (Santa Cruz Biotechnology); ab14545 (1:1000)— β III-tubulin (Abcam, Cambridge, MA); Mab377 (1:100)—NeuN (Chemicon, Billerica, MA); Mab2F11 (1:100)—70-kDa neurofilament protein (Dako, Carpinteria, CA); DAK-SYNAP (1:50)—synaptophysin (Dako). Tissue sections were visualized using a Zeiss Axiovert 200 microscope and photomicrographs were captured using an AxioCam high-resolution CCD camera and Axiovision imaging software (Carl Zeiss International, Germany).

Results

Ectopic expression of *Myc* induces LCA medulloblastomas in *Tp53*–null mice

Considering the fact that LCA medulloblastomas can form when *Myc*-expressing, *Tp53*-deficient neural progenitor cells are implanted orthotopically in immunocompromised mice, we asked whether histologically similar tumors could arise endogenously in response to the same genetic events. To answer this question, we used the above-described RCAS/*tv-a* somatic cell gene transfer system, which makes it possible to transfer and express genes ectopically in Nestin⁺ neural progenitor cells in living mice. To test the effects of both *Myc* overexpression and *Tp53* loss, we generated mice having the combined genotype *Ntv-a/Tp53*^{+/-} and then injected the cerebella of progeny of *Ntv-a/Tp53*^{+/-} parents with an RCAS vector carrying the *Myc* gene (RCAS-*Myc*). Animals showing signs of neurological impairment or general debilitation were sacrificed immediately. All remaining mice were sacrificed for analysis 12 weeks after injection. To assess tumor formation, the brains were dissected, sectioned, and stained with hematoxylin and eosin (H&E). We also analyzed normal DNA from mice to correlate tumor incidence with *Tp53* genotype.

We observed tumors in the cerebellum in 3 of 41 mice (7%). Importantly, tumors formed only in *Tp53*^{-/-} mice (Table 1). No tumors developed in *Ntv-a/Tp53*^{+/+} mice that were injected with RCAS vectors carrying either wild-type *Myc* or a mutant *Myc* allele encoding an amino acid substitution (T58A) that confers resistance to intracellular degradation of the *Myc* protein [15,16].

All tumors showed histological features typical of LCA medulloblastomas. The tumor cells contained large nuclei (11–13 μ m in diameter) and prominent nucleoli (Fig. 1A). This pattern contrasts sharply with the classical cytoarchitecture of *Shh*-induced medulloblastomas in mice, which is characterized by densely packed sheets of cells with small, hyperchromatic nuclei (6–7 μ m) (Fig. 1B). Immunoperoxidase staining showed abundant expression of synaptophysin, a marker for medulloblastoma widely used by surgical pathologists (Fig. 1C) [17]. The tumor cells also showed immunoreactive staining for β III-tubulin in the cytoplasm (Fig. 1D) and NeuN in the nucleus (Fig. 1E). During embryonic development, both of these proteins are expressed by neural progenitor cells at the onset of neuronal differentiation [18,19]. Neurofilament protein, a marker for postmitotic neurons, was expressed in only scattered tumor cells (Fig. 1F). The expression of neuronal markers supports either an origin of the induced tumors from neuronal precursors or a stem cell origin with preferential differentiation along a neuronal lineage.

Although tumors formed in 50% of *Tp53*^{-/-} animals (3/6), we saw three practical shortcomings of using the *Ntv-a/Tp53*^{+/-} strain to create a mouse model of *Myc*-driven LCA medulloblastomas. First, tumors arise only in *Tp53*^{-/-} mice. Second, germline transmission of the defective *Tp53* allele does not follow a Mendelian distribution. In the breeding colony of mice not used for tumor induction experiments, we found that among 76 viable offspring of heterozygous (*Tp53*^{+/-}) parents, only 6 (8%) showed the *Tp53*^{-/-} genotype, rather than the expected 25%. This developmental selection has been reported previously [12]. Third, an alternative breeding strategy using *Tp53*^{-/-} parents cannot be used to increase the transmission of the *Tp53*–null allele because *Tp53*^{-/-} mice are infertile. These factors reduce

the efficiency of this model for generating sufficient numbers of tumor-bearing mice for large-scale gene discovery or preclinical therapeutic testing studies. Adding to the above-described methodological problems, models of *Myc*-driven medulloblastomas in *Tp53*-deficient mice are weakened by the absence of *Tp53* mutations in human Group 3 tumors [20].

Bcl-2 overexpression substitutes for *Tp53* loss to induce LCA medulloblastomas in cooperation with *Myc*

An established paradigm in cancer biology is that oncogene-stimulated cell proliferation cannot initiate tumor formation unless apoptosis is disabled [21]. This phenomenon is explained by an intrinsic, fail-safe mechanism whereby genetic events that stimulate cell cycle progression, like overexpression of *Myc*, sensitize cells to self-destruct by triggering apoptosis. This concept can explain our inability to induce tumors by overexpressing *Myc* in mice with intact *Tp53* because oncogene-induced apoptosis is often mediated by upregulated expression of *Tp53* [22]. As a surrogate for *Tp53* loss, we expressed *Bcl-2*, a general and potent suppressor of apoptosis that is highly expressed in human medulloblastomas, in combination with *Myc* by RCAS transfer in *Ntv-a* mice [23,24]. Tumors developed in the cerebellum of 8 of 44 *Ntv-a* mice (18%) that were injected with RCAS-*Myc* and RCAS-*Bcl-2* and observed for 4 months (Table 1). This tumor incidence was comparable to that in a control group of *Ntv-a* mice injected with RCAS-SHH and observed for the same length of time (25%).

The histopathological features of *Myc+Bcl-2* tumors were similar to those of *Myc*-induced tumors in *Tp53*^{-/-} mice (hereafter called *Myc-Tp53* tumors). The *Myc+Bcl-2* tumors were composed of embryonal cells, some with prominent nucleoli (Fig. 2A). In *Myc-Tp53* tumors, however, tumor cell nuclei were larger, nucleoli were more prominent, and mitotic figures were more common, with some cases showing confluent areas of apoptosis (Fig. 2B). Both *Myc+Bcl-2* and *Myc-Tp53* tumors were highly invasive, spreading to the periventricular parenchyma in the cerebellum and forebrain (Fig. 2C–D). *Myc-Tp53* tumors that spread to the forebrain were less cellular than *Myc+Bcl-2* tumors, but mitotic figures and prominent nucleoli were still common (Fig. 2D inset).

To verify in vivo expression of the genes transferred via RCAS vectors, we performed immunoperoxidase staining of brain tissue sections containing *Myc+Bcl-2* tumors (Fig. 3A) with antibodies directed against the encoded Bcl-2 and Myc proteins, both of which were derived from human sequences. To detect expression of retrovirus-transferred *Bcl-2*, we used monoclonal antibody Mab100, which specifically detects the human Bcl-2 protein and does not cross-react with endogenous mouse Bcl-2. To detect retroviral *Myc*, we used monoclonal antibody 9E10, which binds an epitope present in the human Myc protein, but not the mouse protein. We observed Bcl-2 staining in the cytoplasm (Fig. 3B) and Myc staining in the nuclei of tumor cells (Fig. 3C), consistent with the normal cellular localization of these proteins.

Medulloblastomas induced by *Myc*+*Bcl-2* and *Myc*–*TP53* disseminate frequently to the spinal leptomeninges

Considering the high rate of metastatic dissemination in patients with *Myc*-driven, Group 3 medulloblastomas, we expected to find a high incidence of spinal leptomeningeal dissemination in mice bearing *Myc*+*Bcl-2* tumors and *Myc*–*TP53* tumors. Examination of H&E-stained spinal cord sections from *Myc*+*Bcl-2* mice showed clusters of tumor cells attached to the leptomeninges of the cord or transiting spinal nerves (Fig. 3D) in 7 of the 8 mice with histologically verified brain tumors (87%). The incidence of spinal leptomeningeal dissemination in *Myc*+*Bcl-2* tumors was ten-fold higher than that in *Shh*-induced tumors (8%; $P<0.001$ by Fisher's exact test) (Table 1). Spinal dissemination was also prevalent in *Myc*–*TP53* tumors, occurring in 2 of 3 tumor-bearing mice (66%; $P=0.081$ compared with SHH). The lack of statistical significance of the latter comparison reflects the small number of animals with *Myc*–*TP53* tumors, an inherent limitation of the *Ntv-a/TP53*^{+/–} mouse strain. Immunoperoxidase staining of spinal cord sections showed expression of retrovirus-transferred *Myc* and *Bcl-2* in the disseminating tumor cells (Fig. 3E, F), indicating that these genes conferred metastatic competence to cells in the primary tumor.

Myc and prosurvival *Bcl-2* family members are coexpressed in human Group 3 medulloblastomas

To validate the relevance of our *Myc*+*Bcl-2* model to human medulloblastoma, we evaluated the expression of *Myc* and prosurvival *Bcl-2* family members *Bcl-2*, *Bcl-X_L*, *Bcl-W*, *Mcl1*, and *Bcl-B* (reviewed in [25]) across a discovery cohort (n=46) and validation cohort (n=51) of human Group 3 tumors, which had been profiled previously on Affymetrix Gene 1.1ST and Affymetrix HG-U133A arrays, respectively [26,2]. We performed a z-score analysis to identify correlations between expression of *Myc* and the prosurvival *Bcl-2* family members (Supp. Fig. 1). Expression of both *Myc* and *Bcl-2* was higher than the mean (z-score>0) in 25% and 22% of Group 3 tumors from our discovery and validation cohorts, respectively. Interestingly, some tumors with relatively low *Bcl-2* expression (z-score<0) showed increased expression of different prosurvival genes. Taken together, these results indicate that while *Myc* and *Bcl-2* are coexpressed in Group 3 medulloblastomas, upregulated expression of *Bcl-2* may not be the only mechanism utilized by tumor cells to escape *Myc*-induced apoptosis.

Discussion

Here we report a new mouse model of *Myc*-driven medulloblastoma, in which tumors arise in situ after retroviral transfer and expression of *Myc* in Nestin⁺ neural progenitor cells in the cerebellum of newborn mice. Tumor induction required concomitant loss of *TP53* or overexpression of the antiapoptotic (prosurvival) protein *Bcl-2*. Like *Myc*-driven medulloblastomas in humans, the tumors induced in mice by *Myc*+*Bcl-2* and *Myc*–*TP53* showed LCA cytoarchitecture and a high rate of metastatic dissemination to the spine.

Other investigators have shown that *Myc*-expressing, *TP53*-deficient mouse neural progenitor cells form LCA medulloblastomas when implanted orthotopically in immunocompromised hosts [9,10]. The gene expression profiles of the engrafted mouse

tumors closely match those of human Group 3 medulloblastomas, showing increased expression of known *Myc* transcriptional targets and decreased expression of genes associated with neuronal differentiation. Compared with these transplantation models, in which tumors develop from an inoculum of 10^5 – 10^6 *Myc*-expressing cells, tumor induction in our model system is less efficient. Prior work has shown that in the mouse brain only a few hundred cells at the injection site can be transduced by RCAS retrovirus [27]. Moreover, transduction rates are very low, ranging from <1% to 20%—reviewed in [28]. Nevertheless, our approach of inducing tumors by oncogene transfer to the limited number of somatic cells located at the injection site more closely mimics sporadic tumorigenesis, in which a single transformed cell undergoes clonal expansion within its native microenvironment. Furthermore, the fact that tumors in our model developed under conditions of intact host immune surveillance will make it possible to use our model for testing immunotherapy. The observation that human medulloblastomas express highly immunogenic cancer testis antigens offers one set of attractive immunotherapy targets [29–31].

The dependence of *Myc*-driven tumorigenesis on a cooperating cell survival signal provided by *Bcl-2* expression or *Tp53* loss can be explained by the apoptosis-inducing function of *Myc* and other oncogenes. In support of this explanation, we reported previously that suppressing apoptosis by overexpressing *Bcl-2* enhances the formation of *Shh*-induced medulloblastomas and platelet-derived growth factor-induced gliomas in mice [14,32]. Alternatively, other cell biological functions of *Tp53* and *Bcl-2* might be involved. The apoptosis-independent effects of *Tp53* have been known for many years. Recent research has uncovered functions of *Bcl-2* beyond its canonical role in inhibiting cell death programs—reviewed in [33]. For example, upregulated expression of *Bcl-2* inhibits DNA repair systems, leading to genomic instability through molecular mechanisms that remain incompletely elucidated.

Our finding that *Myc* and *Bcl-2* are coexpressed in 25% of human Group 3 medulloblastomas is consistent with the results of an immunohistochemistry study, published prior to molecular subtyping and showing abundant Bcl-2 protein in 33% of medulloblastomas from patients younger than 6 years [24]. The association between early age of onset and Group 3 medulloblastomas supports the idea that *Bcl-2* expression is upregulated in this molecular subgroup. The prospect that Bcl-2 might be a feasible therapeutic target is supported further by promising results from a preclinical study showing sensitivity of human medulloblastoma cell lines to obatoclax, a drug that inhibits multiple Bcl-2 family members [34].

The distinctive tumor cell morphology of LCA medulloblastomas is likely the direct result of the cell biological functions of *Myc*, one of which is the ability to positively regulate ribosome biogenesis by enhancing transcription of ribosomal RNA, structural ribosomal proteins, and translation initiation factors—reviewed in [35]. *Myc*-stimulated ribosome biogenesis can explain enlargement of tumor cell nucleoli, which are the nuclear sites of a cell's ribosome machinery. The overall size of cells is also determined by *Myc*. Increased expression of the *Drosophila melanogaster* homolog of *Myc* increases the size of whole cells as well as the nuclei and nucleoli of individual cells [36]. In mice, transgenic

overexpression of *Myc* increases the size of hepatocytes and lymphocytes and expands their component nuclei and nucleoli [37,38].

Genetically engineered mouse models reported by other laboratories have shown that tumors resembling LCA medulloblastomas can result from altered expression of different sets of genes in different populations of cells. RCAS retrovirus–targeted expression of *Myc* in combination with the gene encoding β -catenin, a component of the WNT signaling pathway, in cells expressing the astrocyte marker glial fibrillary acidic protein (GFAP) induces tumors with the LCA histological pattern [39]. In a different mouse model, LCA medulloblastomas were generated when both *Tp53* and the retinoblastoma tumor suppressor gene *Rb* were simultaneously knocked out via Cre recombinase in GFAP⁺ cells [40].

Kaplan-Meier analysis did not show a significant survival difference between mice bearing *Myc+Bcl-2* tumors and those with SHH-induced tumors over a 4-month observation period (Supp. Fig. 2). This finding seems paradoxical in view of the high rate of spinal dissemination in mice with *Myc+Bcl-2* tumors and the close association between shortened patient survival times and overexpression and amplification of the *Myc* gene [41]. One interpretation of the mouse survival statistics is that *Myc+Bcl-2* tumors have the same growth rate as SHH tumors, at least at the primary site in the cerebellum. In our study, survival time was determined by growth of the primary tumor, not by metastatic disease. That is, we sacrificed tumor-bearing mice because they developed symptoms arising from brain stem compression or obstructive hydrocephalus, which we did not attempt to treat. This experimental approach contrasts sharply with clinical practice, in which aggressive surgical resection of the primary tumor and decompression of hydrocephalus are essential for progression-free survival in patients.

The high rate of spinal dissemination in mice with *Myc*–induced tumors indicated that *Myc* was conferring cell traits that enable medulloblastoma cells to detach from the tumor mass, enter the cerebrospinal fluid (CSF), and seed the leptomeninges. The ability of cells to proliferate without attachment to a solid surface (anchorage-independent growth) would seem to be an essential attribute of metastasizing medulloblastoma cells, which must survive passage through the CSF before implanting on the pial surfaces of the spinal cord. In support of this concept, ectopic expression of *Myc* enables human medulloblastoma cell lines to form colonies efficiently in soft agar, a measure of anchorage-independent growth [42]. Furthermore, we reported previously that genes that promote leptomeningeal dissemination in mice with *Shh*–induced medulloblastomas confer anchorage independence to medulloblastoma cells in culture [43].

The incomplete penetrance of tumor formation in the *Myc+Bcl-2* model (18%) can be exploited to identify other genes that cooperate with *Myc* to enhance tumor formation. The RCAS/*tv-a* system is ideally suited to identifying cooperating oncogenes because multiple different genes can be transferred and expressed simultaneously. The high rate of spinal dissemination will be an asset for experimental therapies that target the most lethal aspect of medulloblastoma pathogenesis. Although leptomeningeal dissemination is responsible for virtually 100% of medulloblastoma deaths, it remains the most poorly understood component of the disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Histopathology of *Myc-Tp53* medulloblastomas. *A*, Large cell anaplastic cytoarchitecture of *Myc-Tp53* tumor. Tumor cells have large nuclei, some with prominent nucleoli. *B*, Classical cytoarchitecture of mouse medulloblastoma induced by retroviral transfer and expression of *Shh*, showing homogeneous sheets of cells with small, carrot-shaped nuclei and scant cytoplasm. *C–F*, Immunocytochemistry of *Myc-Tp53* medulloblastomas, showing expression of synaptophysin (*C*), β III-tubulin (*D*), and NeuN (*E*) in the majority of tumor cells. Scattered tumor cells showed perinuclear staining for neurofilament protein (*F*). Scale bar, 16 μ m.

Figure 2.

Comparison of histopathological features of *Myc+Bcl-2* and *Myc-Tp53* medulloblastomas. *A*, LCA pattern of *Myc+Bcl-2* medulloblastoma. *B*, LCA features of *Myc-Tp53* medulloblastoma. Asterisks show foci of confluent apoptosis. *C–D*, Invasion of the subependymal region of the lateral ventricle of the forebrain by *Myc+Bcl-2* (*C*) and *Myc-Tp53* (*D*) tumor cells. Ciliated ependymal cells mark the ventricular lining. High-magnification inset shows mitotic figures (arrowhead) and prominent nucleoli (arrow). Scale bar, 25 μm (*A–B*), 50 μm (*C–D*), and 16 μm (inset).

Figure 3.

Expression of RCAS retrovirus–transferred *Bcl-2* and *Myc* in primary (cerebellar) tumors and spinal metastases. *A*, Primary tumor abutting the fourth ventricle of the cerebellum. *B–C*, Immunoreactive staining for *Bcl-2* in the cytoplasm (*B*) and *Myc* in the nuclei (*C*) of tumor cells in primary cerebellar tumor. *D*, Aggregate of tumor cells attached to the pial surface of the spinal cord. A transiting spinal nerve is visible below. *E–F*, Cytoplasmic *Bcl-2* staining (*E*) and nuclear *Myc* staining (*F*) in spinal metastasis. Antibody probe Mab100 is specific for retrovirus–transferred human *Bcl-2* and does not cross-react with mouse *Bcl-2*. Antibody 9E10 reacts specifically with human c-Myc epitope EQKLISEEDL, which is not present in the homologous mouse protein. High-magnification insets show cellular detail. Scale bar, 50 μm (*A–F*) and 16 μm (insets).

Table 1Incidence of medulloblastoma formation in *Ntv-a* mice after somatic cell gene transfer

Genes transferred	<i>TP53</i> genotype	Brain tumor incidence*	Spine tumor incidence*	<i>P</i> value [†] (spine)
<i>Myc</i>	+/+	0 of 16	–	–
<i>Myc</i>	+/-	0 of 19	–	–
<i>Myc</i>	-/-	3 of 6 (50%)	2 of 3 (66%)	0.081
<i>Myc</i> + <i>Bcl-2</i>	+/+	8 of 44 (18%)	7 of 8 (87%)	<0.001
<i>Shh</i>	+/+	12 of 48 (25%)	1 of 12 (8%)	–

* Spine tumor incidence calculated as a percentage of mice with histologically verified brain tumors.

[†] *P* values were calculated using Fisher's exact test to compare spine tumor incidence after combined gene transfer versus transfer of *Shh* alone.