Embryonic Retinal Tumors in SV40 T-Ag Transgenic Mice Contain CD133+ Tumor-Initiating Cells

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PURPOSE. Human retinoblastomas form during the proliferative phase of retina development and are caused by mutations that result in absent or functionally defective Rb protein. Similar tumors occur in mice only when multiple Rb gene family members are absent. We asked if retinal tumors can arise from an undifferentiated retinal cell. The tumor-initiating cells isolated from these tumors that formed in early embryonic murine retinas were characterized.

METHODS. Transgenic mice were created using a *Pax6* promoter to target expression of SV40 large T-antigen (T-Ag) in the undifferentiated murine embryonic retina. T-Ag, which sequesters all Rb family proteins and p53, is expressed in the retina and lens by murine embryonic day 10 (E10) and tumors are observed by E12.5. A cell line that is adherent in serum-containing media and forms neurospheres in supplemented serum-free media was developed from retinal tumors isolated on postnatal day 7.

RESULTS. In all, 1.5% of attached cells form neurospheres when transferred to serum-free medium. All cultured cells express T-Ag, confirming that they derive from the original tumors; 0.5% of adherent cells express detectable levels of CD133. CD133+ FACS-sorted cells cultured in serum-free medium form 3-fold more neurospheres than do CD133- cells. Six of seven mice injected with CD133+ cells and one of seven mice injected with CD133- cells formed tumors during a 6-month period. Unlike primary adherent cells, primary and secondary tumors

heterogeneously express markers of stem cells and differentiation similar to human retinoblastoma.

CONCLUSIONS. CD133+ tumor-initiating cells can originate from proliferating undifferentiated precursor cells. (*Invest Ophthalmol Vis Sci.* 2012;53:3454-3462) DOI:10.1167/iovs.12-9549

Transgenic murine models are being used to investigate whether tumor cells of origin are proliferating progenitor cells or differentiated cells that dedifferentiate into proliferating cells. One such transgenic model of retinoblastoma suggests that a terminally differentiated retinal horizontal cell can dedifferentiate and form retinal tumors.¹ The present study addresses the question whether a proliferating, undifferentiated retinal precursor cell can initiate tumors and, if so, can a subpopulation of these cells that can recapitulate the primary tumor be identified.

Tumor-initiating cells appear to share some properties of stem cells and are defined by their ability to self-renew, to produce heterogeneous progeny, and to exhibit in vitro clonogenicity and in vivo tumorigenicity. They are likely to be the cells driving the growth, progression, and recurrence of a tumor.²⁻⁵ Tumor-initiating cells are involved in the development of several types of malignancies.⁶⁻¹⁰ Most tumors exhibit heterogeneous phenotypes.^{11,12} Expression of CD133 has been used as one criterion to identify tumor-initiating cell populations.^{13,14} Diverse subpopulations of cells within a tumor can have a significant impact on treatment strategies; tumor-initiating cell populations have been shown to be resistant to conventional antitumor therapies.^{15,16} Isolating this population and understanding its biological processes could lead to more effective therapeutic strategies for retinoblastoma and other tumors.

Retinoblastoma occurs naturally only in human children and results from loss of function of both *RB1* alleles. The tumor is heterogeneous, containing biochemical and morphologic evidence of different retina cell phenotypes, including glial and photoreceptor elements.^{17,18} There are minimal data pertaining to the presence of retinal tumor-initiating cells in human retinoblastoma.¹⁹ Retinoblastoma tumors and cell lines have been reported to express proteins common to neural stem cells²⁰ and terminally differentiated murine retinal horizontal cells also express progenitor cell markers.¹ However, the complete characterization of human retinal tumor-initiating cells derived from primary retinoblastoma tumors and the description of their tumorigenic potential is lacking. To facilitate understanding of the tumor-initiating process, transgenic murine models of retinoblastoma have been developed. Mutation of the *RB1* gene is not sufficient to form retinal tumors in mice,²¹ necessitating the use of either simian virus 40 (SV40) large T-antigen (T-Ag) to sequester p53 and all Rb family members (Rb, p107, p130)²²⁻²⁴ or knockout of multiple Rb family members to develop murine models. Various eye-specific promoters have been used, but when the promoter becomes active and in what

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cell population the promoter is expressed dictate when the tumor will form and what cell type initiates the tumor.

Pax6, considered a master regulator gene of eye development, is expressed in retinal progenitor cells as early as embryonic day 9.5 (E9.5)²⁵ prior to retinal differentiation²⁶ and also drives later retinal development.²⁷ A transgenic murine model of retinoblastoma using the *Pax6* promoter to drive the expression of T-Ag was developed. We speculated that tumors would develop from proliferating retinal cells to form retinal tumors similar to human retinoblastoma. We found a subpopulation of the retinal tumor cells that expresses CD133 in culture and is capable of forming heterogeneous tumors similar to the primary retinal tumor when transplanted in vivo.

MATERIALS AND METHODS

Development of Transgenic Mice and Cell Line

All procedures using animals were conducted using protocols approved by the Baylor College of Medicine Institutional Animal Care and Use Committee and adhere to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Transgenic mice expressing SV40 large T-Ag driven by the *Pax6* promoter were established. The *Pax6* enhancer/promoter was linked to a 2.7-kb fragment of DNA containing the entire early region of SV40. The Pax6-T-Ag sequences were isolated as a 5-kb band after digestion with *SpeI* (New England Biolabs, Ipswich, MA) and microinjected into one-cell stage FVB (Friend Leukemia Virus, strain B) embryos. Seven transgenic founders were obtained, six of which expressed the T-Ag and developed ocular tumors. Three founders were used to establish stable families. Tumors were harvested from the OVE1796 family of mice at postnatal day 7 (P7) and used to establish a murine retinoblastoma cell line RTM1796.

Cell Culture

The RTM1796 cells were grown either as neurospheres in a proprietary medium optimized for neural stem cell culture (NeuroCult NSC Basal Medium [Mouse]) supplemented with serum-free defined supplements (NeuroCult Proliferation Supplements [Mouse]) and recombinant human epidermal growth factor (rhEGF, 20 ng/mL) (STEMCELL Technologies, Vancouver, BC) or as a monolayer in Dulbecco's modified Eagle's medium (DMEM)/F12 50/50 medium with 6 g/L glucose, 2 mM L-glutamine, 55 mg/L sodium pyruvate (Mediatech, Manassas, VA), 10% (vol/vol) heat-inactivated fetal bovine serum (FBS; Gemini Bio-Products, West Sacramento, CA), and 1% (vol/vol) penicillin-streptomycin liquid (10,000 IU/mL penicillin G sodium, and 10 mg/mL streptomycin sulfate in 0.85% saline; Mediatech). The cells were maintained in a 37°C humidified incubator with an atmosphere of 95% air and 5% CO₂. All cultures were negative for mycoplasma as determined by DNA staining.

Self-Renewal Assay

A single-cell suspension of a randomly selected RTM1796 neurosphere was prepared by repeated pipetting. Cells of uniform size were selected using a fluorescence-activated cell sorter (FACS, MoFlo) and a single cell was plated into each well of a 96-well culture dish. The cultures were maintained with fresh media weekly and monitored for neurosphere growth for 4–6 weeks.

Secondary Tumors

Exponentially growing RTM1796 cells cultured as a monolayer in serum-containing medium were harvested, washed, and resuspended in serum-free DMEM/F12 medium. Viable cells (2×10^4 by trypan blue exclusion) were injected into the vitreous of *Rag2*($-/-$) immune-incompetent mice.

Immunocytochemistry and Immunohistochemistry

Tumors were harvested, fixed in 10% formaldehyde, and paraffin-embedded for analysis by hematoxylin/eosin staining or by immunohistochemistry. Mouse RTM1796 cells were grown on slides, air dried, and fixed in 2% formaldehyde/PBS for 20 minutes. Fixed slides were washed with PBS and the cells were permeabilized in 0.2% Triton X-100 (Sigma, St. Louis, MO) and analyzed using immunocytochemistry. Paraffin-embedded or frozen tissue sections were processed for immunohistochemistry analysis using antibody-specific antigen retrieval kits (Dako, Carpinteria, CA). The mouse monoclonal antibodies used included antibodies to SV40 T-Ag (EMD Millipore, Billerica, MA), CD133 (prominin; Miltenyi Biotech Inc., Auburn, CA), neuronal-specific enolase (NSE; Dako), and synaptophysin (Dako). The rabbit polyclonal antibodies used were specific for Sox2 (EMD Millipore) and nestin (Abcam, Cambridge, MA). Immunocytochemistry and immunohistochemistry were performed in accordance with standard protocols with the listed antibodies. Images were acquired using a phase-contrast light microscope (Nikon, Melville, NY), with a $\times 40$ objective and potential differences in immunohistochemistry between cells differentially sorted for CD133 expression and unsorted cells were analyzed. For each marker, the percentage of tumor section expressing each antigen, excluding areas free of tumor and normal retina structures, was calculated using ImageJ software (NIH Image, developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at <http://rsb.info.nih.gov/ij/index.html>). Values obtained from images from extraocular and intraocular loci within the tumor were averaged and statistical analysis for significance between the groups was performed using the unpaired two-tailed *t*-test. Values from tumors within the unsorted, sorted CD133+, and sorted CD133– groups were averaged and statistical analysis for significance between the sorting groups was performed using the Kruskal-Wallis ANOVA test coupled with Dunn's multiple comparison post test.

Immunophenotyping and Flow Cytometry

RTM1796 cells (1×10^6) grown in serum-containing medium were washed in PBS and counted. Cells were incubated with CD133 antibody (BD Biosciences, San Jose, CA) or CD15 antibody (BD Biosciences) at 4°C for 10 minutes, washed three times in PBS, and resuspended in fresh PBS for flow cytometric analysis. Cells were collected after sorting and used for neurosphere assays or for in vivo transplantation. Preincubation of the RTM1796 cells with an antibody to the Fc receptor did not alter the results.

Neurosphere Assays

CD133+ or CD133– cells in defined stem cell media were plated in triplicate 24-well culture plates (10^3 – 10^5 cells/well). Cells were incubated at 37°C for 48 hours. Neurospheres formed were manually counted. Differences in the number of neurospheres formed in each of the CD133+, CD133–, and unsorted groups were analyzed for statistical significance using one-way ANOVA coupled with the Tukey multiple comparison post test.

Transplantation of CD133-Sorted Cells

RTM1796 cells were sorted for CD133 expression and injected into the vitreous of *Rag2*($-/-$) mice at a dose of 2×10^3 cells per mouse. Mice were monitored daily for signs of tumor growth. Kaplan-Meier event-free survival curves were created and CD133+ and CD133– groups were compared using the Mantel-Cox log-rank test.

RESULTS

Creation of a Murine Model of Retinoblastoma

To create a murine model of retinoblastoma that develops when the undifferentiated retina is still proliferating, a *Pax6*

promoter was used to drive the expression of the SV40 large T-Ag in the developing undifferentiated embryonic retina. T-Ag expression was documented at E12.5 (Fig. 1A). At E12.5, 100% of OVE1796 transgenic mice developed retinal and lens tumors that rapidly progressed throughout the fetal and postnatal life (Fig. 1B). These animals fail to develop evidence of retinal differentiation. Similar to human disease, the retinal-derived tumors invade the choroid and metastasize through the optic nerve to the brain (Fig. 1C).

Generation of the Murine RTM1796 Cell Line from the Primary Tumor

Primary retinal tumor cells harvested from OVE1796 mice at P7 were grown in defined serum-free stem cell medium or DMEM/F12 supplemented with 10% FBS. Cultures grown in defined medium grew as neurospheres, whereas cultures grown in DMEM/F12 grew as an attached monolayer in the culture plate (Fig. 2A). The population doubling time of tumor cells grown in DMEM/F12 was 19.4 ± 1.4 hours. When the DMEM/F12 was replaced by defined medium, 1.5% of the attached cells plated at a concentration of 1×10^4 cells per mL formed neurospheres of uniform size. To determine if a population of cells capable of self-renewal exists within the neurosphere, cells from neurospheres growing in defined medium were dispersed by pipetting and plated by FACS into 96-well culture plates, one cell per well. When cultured in defined medium for 96 hours, single neurospheres were found in 10% of the wells (Fig. 2A). The cycle of neurosphere dispersal and redevelopment can be repeated (data not shown).

To verify that the isolated cells maintain T-Ag expression, the cultured cells from the mouse tumor were immunocytochemically examined for the expression of T-Ag. All of the cultured cells examined, whether in DMEM/F12 (Fig. 2B) or in defined medium (Fig. 2C), expressed T-Ag. T-Ag negative cells were not observed using any of the culture conditions.

Cultured RTM1796 cells grown in DMEM/F12 were sorted for CD133 expression by FACS. Only 0.4–0.9% of the cells were CD133+. All cells, whether unsorted, CD133+, or CD133– grown in DMEM/F12 uniformly expressed the stem cell markers Sox2 and nestin (Fig. 3A).

Cultured Tumor Cells Expressing CD133 by FACS Have Self-Renewal Properties In Vitro and In Vivo

After sorting, the cells were cultured in defined medium (1×10^5 cells/well) and the number of neurospheres was quantified after 48 hours. Significantly more neurospheres were found to form from CD133+ cells than from CD133– or unsorted cells ($P < 0.0001$, Fig. 3B). When 2×10^3 CD133+ or CD133– cells were injected into the vitreous of *Rag2*(–/–) mice, tumors formed in six of seven mice injected with CD133-expressing cells and only one of seven mice injected with CD133– cells ($P = 0.006$, Fig. 3C). Therefore, the CD133+ population is more likely to initiate neurospheres in vitro and ocular tumors in vivo. CD15 has been found to be expressed on murine brain tumor-initiating cells²⁸ but was not found to be present on the murine retinoblastoma cells (data not shown).

Primary Murine and Human and Secondary Murine Retinoblastomas Have Similar Expression of Neural Stem Cell and Differentiation Markers

Primary OVE1796 murine tumors and human retinoblastoma tumors were immunocytochemically examined for the presence of Sox2 (a transcription factor essential to self-renewal of undifferentiated embryonic stem cells), CD133 (a plasma membrane protein found on neural and endothelial stem cells), and nestin (a class of intermediate filaments expressed on neural stem cells very early during brain development). These proteins have been detected by immunostaining

FIGURE 1. Generation of transgenic mice that develop retinal tumors. (A) Tumors developed and express SV40 T-Ag (brown) by day E12.5. (B, left 2 panels): A normal murine eye with normal developing lens (L), retina (R), and optic nerve (ON) at days E12.5 and P7 compared with the right 2 panels: An OVE1796 murine eye at days E12.5 and P7 showing tumors in the L and R. (C) OVE1796 mouse (P21) with choroidal invasion (C) of tumor (T, left panel), and OVE1796 mouse (P44) with optic nerve (ON) invasion of tumor (arrow, middle panel) and tumor metastases (arrow) to the brain (B, right panel). Scale bars: black bar, 1 mm; red bar, 100 μ m; blue bar, 200 μ m.

FIGURE 2. Culture of primary murine retinoblastoma cells. (A) RTM1796 cells were grown in neural stem cell proliferation media supplemented with epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) growth factor (0.02 $\mu\text{g}/\text{mL}$ and 0.01 $\mu\text{g}/\text{mL}$, respectively) at 37°C and 5% CO_2 until they formed neurospheres after 48–96 hours (*left panel*). Neurospheres were passaged at 48-hour intervals. RTM1796 cells grown in DMEM/F12 medium containing 5% fetal bovine serum grew as a monolayer (*middle panel*). Cells were passaged every 48 hours. A primary neurosphere was disrupted and a single cell per well was plated in a 96-well tissue culture plate using a cell sorter. Neurospheres grew in 10% of the wells plated within 96 hours (*right panel*). (B) Immunolabeling of RTM1796 cells with anti-SV40 T-Ag. Immunocytochemistry was performed on fixed cells. Control cells were developed without SV40 T-Ag antibody (*top panels*). Expression of SV40 T-Ag (*green fluorescence*) was detected in the nucleus of RTM1796 cells (*right bottom panel*) but not observed when the primary antibody was not included (*right top panel*). Hoechst stain was used to counterstain DNA in the nuclei (*left panels*). (C) Neurospheres were fixed, pelleted, paraffin-embedded, sectioned, and probed with antibody against T-Ag (*red*). Scale bars: *black bar*, 100 μm ; *blue bar*, 200 μm ; *white bar*, 200 μm .

frequently in tumor-initiating cells in other cancers as well as in stem cells.^{29–31} Subpopulations of cells within the primary murine and human tumors were positive for CD133, Sox2, nestin, or NSE (a protein in many tumors of neural origin) (Fig. 4A). Similar expression patterns were seen in tumors developed in mice injected with RTM1796 cells or with CD133+ cells. CD133 is expressed in the murine retina in photoreceptors and in cells in the inner retina. Sox2 is expressed in the inner retina and in ganglion cells. Nestin is found in photoreceptors, the inner retina, and the ganglion cell layer. NSE is expressed throughout the retina. Similar to the normal retinal tissue in an E12.5 mouse, the tumors formed in these animals exhibited a high percentage of cells expressing CD133, Sox2, and nestin (Fig. 4B). The percentage of tumor expressing each marker in postnatal mice was quantified using ImageJ software. No difference in the expression patterns could be identified between unsorted and CD133+ sorted secondary tumors and all tumors showed heterogeneous expression patterns for all antigens tested (Fig. 4C). Some of the tumors extended into the extraocular tissue. No difference in expression of these proteins could be found between intraocular or extraocular tumors (Fig. 4D). Therefore, the secondary tumors appeared to recapitulate the primary murine tumors. Unlike the primary cultured RTM1796 cells that homogeneously express nestin and Sox2, both primary and secondary murine tumors have heterogeneous marker expression patterns similar to human retinoblastoma. The tumor that formed in a mouse injected with CD133– cells was similarly examined and exhibited the same characteristics of CD133, nestin, Sox2, and NSE expression as the other tumors (data not

shown). Whether that tumor resulted from a CD133+ cell that failed to be sorted or by a CD133– cell could not be determined, although the existence of CD133+ cells within the tumor favors the former hypothesis.

DISCUSSION

One unresolved issue in cancer biology is whether tumors originate from primitive progenitor cells or from differentiated cells that become malignantly transformed.¹ A related issue is whether tumor-initiating cells maintain a stable “stem-cell” phenotype within a tumor or whether tumors are typically composed of a mixture of cells, only some of which are capable of becoming tumor initiators. One transgenic mouse strain develops retinal tumors arising from dedifferentiated terminally differentiated retinal horizontal cells.¹ This leaves open the question whether human retinoblastoma also results from dedifferentiation of a differentiated human retinal cone cell.^{17,18,32} Because human retinoblastomas express cone phototransduction elements¹⁷ and require a cone-specific MDM2 pathway to proliferate, Xu et al. speculated that a terminally differentiated cone photoreceptor cell dedifferentiates into a proliferating cell that results in retinoblastoma.^{18,32} One would expect that if this were the case, the longer a cell is in the differentiated state, the greater the chance that the genetic and/or epigenetic mutational events necessary for dedifferentiation could occur and that the incidence of retinoblastoma would increase with increasing age. This is contrary to the incidence of retinoblastoma observed in the

FIGURE 3. Isolation of CD133+ cells. (A) Unsorted and sorted RTM1796 cells were shown to uniformly express Sox2 and nestin. DAPI was used to counterstain DNA in the nuclei. (B) Sorted cells were placed in defined medium for 48 hours and the number of neurospheres was determined. There was an increased number of neurospheres formed from the CD133+ population of cells ($P < 0.0001$). (C) To determine if the CD133+ cell population had increased tumorigenicity, 2×10^3 CD133+ or CD133- cells were injected into the vitreous of *Rag2*^{-/-} immune incompetent mice. Tumors formed in six of seven mice injected with CD133+ cells (black), whereas tumors formed in only one of seven mice injected with CD133- cells (red; $P = 0.006$). Scale bars: white bar, 200 μ m.

human disease, which has a decreasing incidence with increasing age and, in fact, the incidence of retinoblastoma parallels the declining ability of the maturing retina to proliferate. Although the study of human retinoblastoma has not yet answered this question, the data presented herein demonstrate that murine retinal tumors can develop from an undifferentiated embryonic retina and these tumors resemble human retinoblastoma. Similar to murine and human brain tumors,^{33,34} a small percentage of cells within the tumor population express CD133 when measured by flow cytometry and these cells can recapitulate the original tumor. Recent observations have questioned whether the CD133+ population of cells represents the sole tumor initiating population of cells within brain tumors.^{35–37} The murine retinal tumor model described herein will help address this question in a retinoblastoma tumor model.

The function of CD133 is not well understood. Published reports implicate CD133 in a variety of cellular pathways, including plasma membrane architecture and organization,³⁸

formation and organization of membrane lipid raft microdomains,³⁹ and regulation of the endocytotic mechanism.⁴⁰ Overexpression of CD133 in a glioma cell line activated pro-growth kinase pathways.⁴¹ In the context of stem cell biology, CD133 expression has been shown to be important in the maintenance of stem cells of multiple lineages.⁴² Additionally, CD133 has been well characterized as a marker of self-renewing tumor-initiating cell subpopulations in neuroendocrine malignancies.^{43–45} However, the specific functional role that CD133 plays, if any, in maintaining stemlike characteristics in tumor-initiating cells is not currently known. In this study, relatively low numbers of RTM1796 cells were detected as CD133+ by flow cytometric analysis; however, subsequent immunostaining shows higher positivity for CD133. The specific reason for this discrepancy is unclear, but published observations suggest that CD133 is heavily glycosylated in its ectodomain region, which can contribute to antigenic variability of common epitopes.⁴⁶ Since proteins are in a denatured state in the paraffin-embedded tissue sections used for

FIGURE 4. Characteristics of primary and secondary murine retinal tumors. (A) Mouse eyes containing primary postnatal tumors and secondary murine retinal tumors created by the injection of either unsorted or sorted CD133+ cells into murine vitreous were compared with normal murine retina and human retinoblastoma for the expression of CD133, Sox2, nestin, and NSE (all brown) using immunohistochemistry. Sections were cut from fixed tissues embedded in paraffin blocks and incubated with each antibody. (B) Normal developing retinal tissue and retinal tumors from E12.5 mice were sectioned from fixed tissues embedded in paraffin blocks and incubated with antibody to CD133, Sox2, and nestin (all brown). (C) The percentage of the tumor area expressing each protein was determined for primary tumors (white), secondary tumors derived from unsorted cells (blue), and secondary tumors derived from CD133+sorted cells (red) using ImageJ software and compared. Although there appeared to be differences in CD133 and NSE expression between primary and secondary tumors, no difference in the expression patterns could be identified between unsorted and CD133+sorted secondary tumors. All tumors showed heterogeneous expression patterns for all antigens tested. (D) Similar analysis of intraocular tumors (white) and of tumors extending into extraocular tissue (black) was performed, and no difference in the expression patterns of the proteins was observed. Scale bars: black bar, 25 μ m; red bar, 40 μ m.

immunohistochemical analysis, antigens that may have been masked in the native state may have been revealed, possibly allowing antibody staining to reveal the true extent of CD133 positivity. Kemper et al.⁴⁷ reported that the antibody-recognized epitopes that are present in cancer stem cell populations are masked upon differentiation, a finding that provides a potential explanation for the discrepancy between CD133 staining by flow and by immunohistochemistry in this study.

Some insight into the characteristics of the TAg-induced murine retinoblastoma cells can be inferred from the RTM1796 cell line. When cultured in serum, the cells grow as an attached population. The cells are uniformly positive for TAg, indicating that they are all of malignant origin. When the attached cells are dispersed and injected into the eyes of mice, the tumors formed are indistinguishable from the original tumor. When the same cell population is analyzed for the ability to form

neurospheres in vitro, only a small number of neurospheres arise, suggesting that only a small percentage of the cells have the capability of self-renewal. The neurospheres share the diversity of differentiation and stem cell markers seen in the primary and secondary tumors. When cells derived from neurospheres are injected into mice, tumors form more rapidly than when attached cells are injected (data not shown). This is consistent with the hypotheses that there are tumor-initiating cells in the attached cell population and that these cells must undergo a change in behavior to become capable of forming neurospheres in vitro or tumors in vivo. Cells that express detectable CD133 are more likely to form neurospheres and tumors than cells that do not. Although the specific function of CD133 regarding maintaining a stem-cell phenotype is not currently known, one could speculate that the regulation of CD133 is either a direct cause or a concurrent effect of control of cell cycle progression and self-renewal pathways. Unlike in humans, mutations in the *RBI* gene alone do not result in retinoblastoma in mice.⁴⁸ This has been postulated to be the result of redundancy in function of the Rb family members during the development of the murine retina. Similar redundancy does not occur in the human fetus.^{49,50} T-Ag inactivates all Rb family members by direct sequestration of the proteins.²² The p53 tumor suppressor protein, which is disrupted in the development of many malignancies, is also bound and sequestered by T-Ag⁵¹ interfering with its activity, and circumventing the Mdm2/MdmX and other degradation pathways. The loss of functioning p53 protein has been shown to be important in self-renewal of stem cells,⁵² suggesting that loss of p53 in this model may be an important precursor for the development and maintenance of CD133+ tumor-initiating cells.

The age of onset of human retinoblastoma correlates with the ability of the retina to proliferate during early childhood. By using the *Pax6* promoter,^{53,54} we were able to express T-Ag in embryonic retinoblasts. Tumors were seen in all animals by E12.5, fulfilling the criterion that the tumors were initiated in the proliferating retina. Similar to the human disease, the tumors can invade the choroid and optic nerve and can metastasize to the brain. Because Pax6 is expressed in ocular tissues other than the developing retina, lens tumors are also found in this animal model; similar tumors do not occur in human children or adults. Importantly, cells from both human⁵⁵ and murine tumors grow as neurospheres in defined medium and heterogeneously express proteins that are found in neural stem cells and differentiated retinal cells. Cell lines derived from human tumors have previously been shown to form ocular tumors when injected into immune-deficient mice.⁵⁶

T-Ag driven by other promoters, including the interphotoreceptor binding protein (IRBP)^{57,58} and β -luteinizing hormone (BLH) promoters,⁵⁹ have been used to initiate murine retinal tumors⁶⁰ and have been used in preclinical studies to evaluate treatment modalities for retinoblastoma.⁶¹ The model described in this study is unique in that the *Pax6* promoter expresses T-Ag in the developing embryonic retina. Because retinoblastoma is uniquely a human disease, these transgenic and xenograft models are critical for the evaluation of the biology and treatment of human retinoblastoma.

Several reports using different mouse knockouts of Rb family genes have speculated that the cell of origin for retinoblastoma may be a stem cell.⁶² Others have questioned this conclusion⁶³ and have identified an already differentiated horizontal cell as a cell of origin of retinoblastoma in a transgenic mouse model of retinoblastoma.¹ Other groups have shown the presence of small populations of cells that express proteins found on neural stem cells in human retinoblastoma tumor cells and in Y79 cultured human retinoblastoma

cells.^{19,20} In our mouse model, the subpopulation of cells that has stem-cell characteristics is of tumor origin, as evidenced by the expression of T-Ag. Most of the normal retinal progenitor and stem cells are known to express early neural progenitor cell markers such as CD133, nestin, and Sox2.⁶⁴ Our data indicate that a tumor-initiating cell expressing these early neural progenitor cell markers in vitro is able to proliferate and generate neurospheres in culture and is capable of forming tumors in vivo that are identical immunohistochemically to the parent tumor. The primary and secondary murine tumors are similar to human tumors and show evidence of differentiation, as evidenced by the expression of the neural differentiation marker NSE in vivo. Therefore the Pax6-T-Ag retinoblastomas contain tumor-initiating cells that are derived from the embryonic, proliferating, developing murine retina and that are capable of initiating and recapitulating retinal tumors similar to those found in the human disease. The finding that retinoblastoma results from a progenitor cell with stemlike characteristics could explain the development of radio-⁶⁵ and chemoresistance⁶⁶ as seen in other malignancies as well as late tumor recurrence seen in the clinical setting. The creation of a murine model that develops retinal tumors and the characterization of tumor-initiating cells in this model should provide valuable tools for the study of characteristics that contribute to the initiation and progression of retinoblastoma.

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