The TAg-RB Murine Retinoblastoma Cell of Origin Has Immunohistochemical Features of Differentiated Müller Glia with Progenitor Properties

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PURPOSE. Human retinoblastoma arises from an undefined developing retinal cell after inactivation of RB1. This is emulated in a murine retinoblastoma model by inactivation of pRB by retinal-specific expression of simian virus 40 large T-antigen (TAg-RB). Some mutational events after RB1 loss in humans are recapitulated at the expression level in TAg-RB, supporting preclinical evidence that this model is useful for comparative studies between mouse and human. Here, the characteristics of the TAg-RB cell of origin are defined.

METHODS. TAg-RB mice were killed at ages from embryonic day (E)18 to postnatal day (P)35. Tumors were analyzed by immunostaining, DNA copy number PCR, or real-time quantitative RT-PCR for TAg protein, retinal cell type markers, and retinoblastoma-relevant genes.

RESULTS. TAg expression began at P8 in a row of inner nuclear layer cells that increased in number through P21 to P28, when clusters reminiscent of small tumors emerged from cells that escaped a wave of apoptosis. Early TAg-expressing cells coexpressed the developmental marker Chx10 and glial markers CRALBP, clusterin, and carbonic anhydrase II (Car2), but not TuJ1, an early neuronal marker. Emerging tumors retained expression of only Chx10 and carbonic anhydrase II. As with human retinoblastoma, TAg-RB tumors showed decreased Cdb11 DNA copy number and gain of Kif14 and Mycn. It was confirmed that TAg-RB tumors lose expression of tumor suppressor cadherin-11 and overexpress oncogenes Kif14, Dek, and E2f3.

CONCLUSIONS. TAg-RB tumors displayed molecular similarity to human retinoblastoma and origin in a cell with features of differentiated Müller glia with progenitor properties. (Invest Ophthalmol Vis Sci. 2011;52:7618–7624) DOI:10.1167/iovs.11-7989

The pediatric intraocular tumor retinoblastoma is initiated by loss of the prototypic tumor suppressor RB1, which encodes the cell cycle regulator pRB.1 However, the specific cell type(s) in the developing human retina dependent on pRB to suppress tumor development remains unknown. The properties of this extremely cancer-susceptible cell are important to understand not only for retinoblastoma but also cancer initiation in general.

Knowledge of the molecular mechanisms responsible for progression of retinoblastoma after RB1 loss has come from retinoblastoma mouse models. Complete knockout of Rb1 in mice is embryonically lethal.5–7 Conditional inactivation of Rb1 and inactivation of additional pRB family members (p107 or p130) are necessary for retinoblastoma development in mice.6–8 Several murine retinoblastoma models use early retinal gene promoters that direct conditional Rb1 inactivation in a subset of retinal cells, combined with constitutional inactivation of p107 or p130. Such approaches have created retinoblastoma models with tumors displaying amacrines and glial cell characteristics.7–9 In each of these models, the specific cell populations affected by Rb1 loss varies and many more cells are rendered Rb1/– than in human disease, where the loss of both Rb1 alleles is a stochastic event in presumably relatively few developing retinal cells.

The simian virus 40 large T antigen (Tag) provides a biochemical means of functionally knocking out pRB family members, along with p53 and other protein targets, and has been used in various mouse tumor models.10 One planned mouse pituitary tumor model was designed to express Tag under the control of the β-luteinizing hormone promoter, but instead developed completely penetrant, heritable retinoblastoma (Tag-RB). Like human retinoblastoma, Tag-RB tumors contain Homer-Wright rosettes and are the only murine retinoblastoma tumors reported to also show Flexner-Wintersteiner rosettes.11 The presence of both types of rosette is a hallmark of human retinoblastoma.12 Because of its high penetrance and histologic11,13 and molecular similarity14–17 to human retinoblastoma, this model has been frequently used by several groups to test retinoblastoma chemotherapies. In fact, approximately 20 studies using this mouse have been published in the past five years alone, spanning drug testing18–27 imaging,28–30 and ba-
sic tumor biology. However, the origin of TAg-RB tumors has not been characterized beyond the discovery that they arise within the inner nuclear layer (INL) of the retina. Less than 1% of TAg-RB tumor cells display markers of stem cells or progenitors, such as ALDH1, SCA-1 and p63.

Here, we have used TAg protein expression in the retina to track tumor development from the earliest stages. We show that the cell of origin belongs to a subpopulation of progenitor-like Müller glia that undergoes transformation upon the expression of TAg.

**METHODS**

**Mice**

Wild type and TAg-RB mice (a gift from the laboratory of Joan O’Brien) were maintained on a pure C57/B6 background and studied using protocols approved by the Animal Care Committee of the Ontario Cancer Institute, in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Timed pregnancies were determined by vaginal plug observation, with midday of plug observation counted as embryonic day (E)0.5.

**Bromodeoxyuridine Incorporation Assay**

Animals were injected (1 ml reagent per 100 g body weight) with bromodeoxyuridine (BrdU) reagent (Zymed, San Francisco, CA) and killed after 2 hours.

**Immunohistochemistry (IHC)**

Formalin-fixed, paraffin-embedded (FFPE) sections (5 μm) of whole embryos, neurons, or adult TAg-RB eyes were studied. For antigen retrieval, sections were treated with 0.1% trypsin for 30 minutes or heated in PBS citrate for 5 minutes in a pressure cooker, followed by 30 minutes in blocking solution (DAKO, Glostrup, Denmark), overnight incubation with primary antibody (Table 1) and 1-hour incubation with biotin-labeled secondary antibody (Table 1) and 1-hour incubation with streptavidin linked to Alexa 488 or Alexa 594. Staining was observed with a microscope (DMLB; Leica, Concord, ON, Canada). Immunoreactivity was detected using a substrate kit (InnvoPure Fab Preparation kit; Pierce, Rockford, IL) and/or fluorescent detection with streptavidin linked to Alexa 488 or Alexa 594. Images were superimposed using image-manipulation software (Photoshop; Adobe, San Jose, CA).

**RNA Analysis**

Total RNA was isolated from wild type C57/B6 mouse retinas and TAg-RB tumors using reagent (TRIZol; Invitrogen, Burlington, ON, Canada), and first-strand synthesis performed with reverse transcriptase (SuperScript II; Invitrogen) according to manufacturer's protocols. Sequence detection (TagMan qRT-PCR) was performed using gene expression assays for Kif14 (Mm01291391_ml), Hprt (Mm00446973_ml) and reference Tbp (Mm00443265_m1). All reactions were performed in triplicate (TagMan Master Mix in a 12.5 μL volume in a 384-well plate in an SDS 7900H; Applied Biosystems, Foster City, CA). RAW PCR cycle number required to reach a fluorescence threshold (G), ΔCt, and ΔΔCt were calculated using commercial software (SDS 2.0; Applied Biosystems).

**DNA Analysis**

Genomic DNA was isolated from 30 TAg-RB tumors and mouse liver (normal control) using a proteinase K and phenol-chloroform extraction method. Semi-quantitative PCR was performed using 50 ng of DNA and polymerase (KOD DNA polymerase; Novagen, San Diego, CA). Reactions were performed for 28 cycles using two primer pairs for each gene: Tbp 5’ (forward: gcctagccacgccagcttact; reverse: tgggtgaggtctgatgtg). Tbp 5’ (forward: ccccaactctccatatcct; reverse: tggttcggccacaggttagat). Cdh11 5’ (forward: acatcgtccagcagcacca; reverse: cagcagcattgagcagctgat). Cdh11 5’ (forward: ctgaagctctctccattcctctg; reverse: ttgctccatctctctctct). Dek 5’ (forward: aggtacgtgagcagcacca; reverse: ggtgtgtgccagaggtggcttt). Dek 5’ (forward: gccacgccagacagctctg; reverse: atctctggttgggtcgtagtct). Hprt 5’ (forward: gccttacgctcctctctctctct; reverse: tggcaggtggagccctctctt). Mycn 5’ (forward: cccactctctctctctctctctct; reverse: cctgtggtcgggctgtagttt). Mycn 5’ (forward: ggtgtgtgccagagggagcag; reverse: gcctctggtcgggctgtagttt). PCR products were visualized on agarose gels and band intensity was analyzed using ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at http://rsweb.nih.gov/ij/). Mean values for the two primer pairs per gene were compared with liver (two-copy control) to calculate gain or loss.

**RESULTS**

**TAG Expression Begins at P8 in the Inner Nuclear Layer of TAg-RB Retina**

We followed the expression pattern of TAg protein in the developing retina of TAg-RB mice by immunohistochemistry (Fig. 1A). TAg expression was first observed in the nuclei of sparse cells within the INL at P8. At P12, an increased number of TAg-positive cells formed a single row of cells in the center of the INL. At P21, TAg-positive cells occupied much of the INL, after which numbers of single cells declined and clusters

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**Table 1. Antibodies Used for Immunohistochemistry**

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<th>Antibody</th>
<th>Marker for</th>
<th>Source</th>
<th>Dilution</th>
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<td>TAg-RB</td>
<td>Santa Cruz, Santa Cruz, CA</td>
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<td>Activated Caspase</td>
<td>Apoptosis</td>
<td>R&amp;D Systems, Minneapolis, MN</td>
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<td>BD Biosciences, Mississauga, ON, Canada</td>
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<tr>
<td>Tuj1</td>
<td>Early neurons</td>
<td>Stemcell Technologies, Vancouver, BC, Canada</td>
<td>1:50</td>
</tr>
<tr>
<td>Ki67</td>
<td>Proliferation</td>
<td>Lab Vision, Fremont, CA</td>
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<tr>
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<td>Amacrine cells</td>
<td>Sigma, Oakville, ON, Canada</td>
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</tr>
<tr>
<td>Chx10</td>
<td>Progenitors/Müller subpopulation</td>
<td>Brenner laboratory&lt;sup&gt;9&lt;/sup&gt;</td>
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<tr>
<td>p75&lt;sup&gt;NTR&lt;/sup&gt;</td>
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<td>DEK</td>
<td>Gained in retinoblastoma</td>
<td>BD Biosciences</td>
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of TAg-positive cells were observed at P28. At P35, multiple large tumor foci were observed. The reduction in TAg-expressing cells from P21 to P28 prompted us to examine whether this decline was due to cell death rather than loss of TAg expression. We double-stained P21 TAg-RB retina for the presence of activated caspase-3 and TAg (Fig. 1B). The majority of cells expressed both proteins; rare cells expressed TAg only (arrow).

**FIGURE 1.** Distinct pattern of TAg expression in the TAg-RB developing retina. (A) Immunohistochemical analysis shows expression of TAg (red) in developing retina from postnatal day (P)5 to P35. Expression of TAg started in single cells at P8, localized to the inner nuclear layer (original magnification, ×400). (B) Expression of activated caspase 3 (green) and TAg (red) at P21, low (×200) (left) and high (×400) (right) magnification. The majority of cells expressed both proteins; rare cells expressed TAg only (arrow).

**TAg-RB Cells Express Proliferation Markers**

Retinal cells from P8 and P9 wild type mice were rarely positive for proliferation markers Ki-67 (stains cells in G1, S, G2, and M phases) or incorporated BrdU (incorporated into DNA during S phase), suggesting that cells had already undergone or were in the process of undergoing terminal mitosis (data not shown). However, in the TAg-RB littermates of these animals, Ki-67 expression and BrdU incorporation were observed in a subset of TAg-positive cells (Fig. 2). At most time points, we observed similar regions of staining for BrdU and TAg, suggesting that the cells in S-phase at the time of BrdU exposure expressed TAg. Interestingly, at P9 and P12 we observed cells that were positive for Ki-67 but not for TAg expression (Fig. 2).

**FIGURE 2.** Expression of proliferation markers in the course of TAg-RB development. Adjacent retinal sections from mice injected with BrdU were immunostained for BrdU (brown), TAg (green), or Ki-67 (red). Although the majority of the cells that were proliferating (BrdU or Ki-67 positive) were TAg-positive, there were a number of cells that showed proliferation marker or TAg expression only. Original magnification, ×400.

**TAg-Expressing Cells Are Negative for Neuronal Marker TuJ1**

To determine whether TAg-expressing cells display neuronal properties, we double stained TAg-RB retinas for TAg and TuJ1, a marker of early neuronal development that is absent in differentiated glia.15,16 TAg-expressing cells were negative for TuJ1 at both early (Fig. 3) and late (data not shown) stages of tumor development.

**FIGURE 3.** TAg expressing cells do not express neuronal marker TuJ1. There was no expression of TuJ1 (green) in TAg (red) positive cells at P9 and P12, suggesting a nonneuronal origin of these cells. Original magnification, ×1000.
Cdh11 copy number for evaluated their role in the TAg-RB model by measuring gene expression in large cells39) (Figures 4, 5, and 6). The same pattern was observed for neurofilament 160 kDa (Nefm) (horizontal cells) or PKC (bipolar cells) (Fig. 4), and clusterin staining in large tumors was inconclusive, but not from TAg-positive cells at P29 and TAg-RB tumors was absent from TAg-positive cells at P29 and TAg-RB tumors (Fig. 4), and clusterin staining in large tumors was inconclusive (data not shown).

Cells positive for both TAg and Chx10 showed less intense Chx10 staining than cells that were positive for only Chx10 (Fig. 4). The double-stained population was confined to a distinct location within the INL, different from the more intensely stained Chx10 population at the external edge of the INL where bipolar cells are normally found. At P9 and P12 the TAg positive cells were negative for PKC, a marker of mature bipolar cells (Fig. 5).

**FIGURE 4.** Expression of TAg overlaps with expression of Müller glial markers. Expression of all major retinal cell type markers (green) and TAg (red) from P9 to P29. No overlap was found for HPC-1 (amacrine cells), or neurofilament 160 kDa (Nefm) (horizontal cells) and complete overlap at all time points was found for Chx10 (retinal progenitors and bipolar cells). Coexpression of CRALBP (Müller glia) and TAg was found only at the very early time point (P9) and by P12, the majority of TAg-positive cells did not express CRALBP. Original magnification, ×1000.

(Tag-RB) Tumors Express Retinoblastoma-Associated Tumor Suppressor Genes during Initiation, and Overexpress Oncogenes during Progression of Tumorigenesis

We have identified a number of genes involved in the progression of human retinoblastoma after loss of RB114–16,40,41. We evaluated their role in the Tag-RB model by measuring gene copy number for Cdb11, Mync, Kif14, E2f3, and Dek in Tag-RB tumors compared with the liver control using semiquantitative PCR (Fig. 7A). Loss of Cdb11 occurred in 45% of Tag-RB tumors. Gain in copy number was evident in Tag-RB tumors for Kif14 (33%), Mync (19%), and E2f3 (18%). Kif14 genomic gain was reinforced at the expression level: Kif14 mRNA was higher in 9 of 11 large tumors than at any point in normal retinal development (Fig. 7B).

Early expression of TAg in single cells at P9 coincided with expression of tumor suppressor genes p75NR14 and Cdh11, and oncogenes Dek and E2f3 (Fig. 7C and data not shown). Expression of E2f3 and Dek remained high throughout tumor development. We have previously shown that expression of the tumor suppressor genes p75NR and Cdh11 is lost from progressing large tumors starting at P28.14,15

**FIGURE 5.** Cells expressing TAg are not mature bipolar cells. Mature bipolar marker PKC (green) showed no overlap with TAg (red) expression. Original magnification, ×1000.

**DISCUSSION**

The Value of the Tag-RB Model

The Tag-RB tumors have striking histologic and molecular similarity to human retinoblastoma, including focal initiation, loss expression of tumor suppressor genes Cdb1115 and p75NR14 overexpression of oncogenes DEK and E2f316 and presence of Homer-Wright and Flexner-Wintersteiner rosettes.11 Approximately 20 different mouse models have been created in an attempt to recapitulate human retinoblastoma (reviewed in Ref. 42), however Tag-RB has the closest histologic resemblance to human tumors. In addition, most other models do not recreate the sporadic nature of human RB1 mutation in a susceptible retinal cell. The Tag-RB model achieves this by inactivating pRB and related proteins in specific cells in the context of an otherwise normal retina.

The functional consequences of TAg expression depend on the cell type and context in which it is expressed; for example, TAg can cause degeneration13–15 or tumor formation15 in photoreceptors and apoptosis in horizontal cells.45 Thus, the expression of TAg in mature neurons does not necessarily lead to oncogenesis. In Tag-RB, TAg expression is governed by an unknown, retinal-specific genomic element that directs expression of TAg to only one cellular population during a narrow window of time during development. Identifying this population was a major goal of the current work.

Regulation of TAg Expression in Tag-RB Mice

Because the expression of TAg was restricted to the INL at all time points studied (Fig. 1), an INL origin of the tumors is certain. TAg expression was tightly regulated developmentally, beginning with only a few TAg-positive cells at P8 and gradually expanding by P13. Active proliferation and the initial steady increase in numbers of Tag-RB cells (Fig. 1) suggest that when TAg is expressed, this trait is maintained in the TAg-expressing lineage. Although at P9, early TAg-expressing cells expressed CRALBP (Fig. 4), later TAg-positive cells and progressing tumors did not express this glial marker.

The majority of TAg-expressing cells also expressed the apoptotic signaling molecule p75NR14 and activated caspase-3 (Fig. 1B) at P21, suggesting that the expression of TAg results in the activation of cell death, accounting for the decrease in Tag-expressing cell number, as we suggested previously.14 Occasional TAg-expressing cells escaped cell death (Fig. 1B, arrow) and formed clusters of early tumors by P28. This defined, clonal regulation of tumor initiation and development, together with complete disease penetrance and retinoblastoma-like histology, justifies the use of the Tag-RB model to test therapies.
The Cell of Retinoblastoma Origin

The cell of retinoblastoma origin is being actively sought (reviewed in Ref. 46). Its identity has been inferred by examining expression of markers of retinal cell type and developmental stage (precursor/stem cell, mature/differentiated) within pathologic specimens of human retinoblastoma. Rod- and cone-specific genes are expressed most frequently, interpreted to indicate that retinoblastoma originates in photoreceptors,47,48 consistent with the presence of rosettes with histologic similarities to photoreceptor outer segments.49,50 Other studies, however, show that retinoblastoma tumors express many other cell type markers, including Müller glia, amacrine, and ganglion cell markers.51,52 To our knowledge, the expression of only one neuronal stem cell marker, MCM2, has been demonstrated in human retinoblastoma, however its expression was mainly restricted to tumor adjacent to blood vessels.35 Although clinical retinoblastoma specimens are invaluable for such studies, they are usually large, advanced tumors necessitating enucleation (retinoblastoma diagnosed early in its development can be successfully treated without enucleation), and often display promiscuous expression of various cell type markers.

Mouse models that use genetic inactivation of Rb1 in combination with p107 or p130 loss develop tumors emerging from the INL that express amacrine and glial cell markers, consistent with observations of the early pRB/p107-deficient chimeric retinoblastoma model.8 As in human retinoblastoma, marker expression in late-stage tumors is promiscuous. Tag is an unambiguous marker of Tag-RB tumor cells, enabling us to follow and analyze characteristics of the cell of origin from the onset of tumor initiation, before transformation could potentially corrupt the normal patterns of gene expression.

The Tag-RB Cell of Origin

We show that the Tag-RB cell of origin displays characteristics of mature Müller glia (Figures 4, 5, and 6). Although gene expression in Müller glia is very similar to that observed in retinal progenitors,50 expression of Müller glial-specific clusterin and carbonic anhydrase II in Tag-expressing cells provides definitive evidence of a Müller glial origin for these cells. The absence of expression of the early neuronal marker TuJ1 (Fig. 3) suggests that the Tag-RB cell of origin is not an early progenitor cell (Fig. 2).

Tag-positive Müller glia expressed Chx10 at a low level from their first emergence at P9 throughout transformation (Fig. 4). This expression was lower in the Tag-expressing cells than in neighboring mature bipolar cells. A subpopulation of postmitotic Müller glia that expresses Chx10 has been identified and proposed as more progenitor-like than non-Chx10-expressing Müller glia.53 Alternatively, it has been proposed that all Müller glia retain neural stem cell properties, and are capable of reversing their differentiation.54 The Tag expression in Chx10-positive cells from the earliest time point studied (P8) is consistent with the cell of origin of the Tag-RB tumors being such Chx10-expressing Müller glia with the capacity of progenitors. Tag could also directly inactivate transcription factors to alter the expression of retinal cell type markers.

We propose that the Tag-RB cell of origin is a sparse, Chx10-expressing differentiated Müller glial cell that is extremely sensitive to Tag oncogenic insult because of its dormant progenitor capabilities. The combination of strong oncogenic stimulation by Tag and the progenitor characteristics of this cell may reduce the minimum number of post-Rb1 loss events needed to achieve malignant transformation. In fully differentiated brain neurons without progenitor features, con-
tuous overexpression of TAg stimulates cell cycle re-entry, followed by cellular degeneration.

**Evasion of Apoptosis and Promotion of TAg-RB Tumorigenesis**

The TAg- and Chx10-positive cells proliferated within the INL of the retina, and by P21 expressed activated caspase-3 (Fig. 1B). We showed previously that the TAg-expressing cells are p75NTR-positive at P21 and display pyknotic nuclei, indicative of apoptosis. Cells that lose expression of p75NTR escape apoptosis and give rise to tumors. By P28, TAg was restricted to foci from which tumors emerge. The activation of apoptosis days after initial TAg expression suggests that a few rounds of uncontrolled proliferation occur without activation of an apoptotic pathway. It has been suggested that the human retinoblastoma cell of origin evades apoptosis by functional inactivation of p53, either by increased MDM2 and/or MDM4 or posttranscriptional inactivation of p14ARF. Because p53 is inactivated by TAg in our model, we could not assess this in our study.

The eventual TAg-RB tumors arose from the less common cells that escape apoptosis. The time delay between initial TAg expression and eventual tumor formation implies the need for the accumulation of other mutations for complete malignant transformation. Indeed, we found that several genomic changes characteristic of human retinoblastoma were also evident in TAg-RB at both the genomic and expression levels (Fig. 7). Our findings complement a recent microarray study of TAg-RB tumors that demonstrates overlap with theactivation of tumor tissue levels (Fig. 1B).

References


