Retinoblastoma: From the Two-Hit Hypothesis to Targeted Chemotherapy

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Abstract

Studies on retinoblastoma have been at the heart of many of the landmark discoveries in cancer genetics over the past 35 years. However, these advances in the laboratory have had little effect on the treatment of children with retinoblastoma. One of the reasons for this has been the lack of preclinical models that recapitulated the genetic and histopathologic features of human retinoblastoma. In the past three years, a series of new animal models of retinoblastoma has been developed and characterized from several different laboratories using a variety of experimental approaches. It is encouraging that there is broad agreement about the consequences of inactivation of the Rb family in retinal development from these studies. More importantly, these new mouse models of retinoblastoma have contributed to clinical trials and novel therapeutic approaches for treating this debilitating childhood cancer. [Cancer Res 2007;67(16):7547-50]

Introduction

In 1971 Knudson proposed that retinoblastoma was initiated by inactivation of a putative tumor suppressor gene (1), and this hypothesis was subsequently confirmed by demonstration of loss of heterozygosity at 13q14 in retinoblastomas (2) and the cloning of the first tumor suppressor gene Rb1 (3). A few years later, Harbour extended these findings to small cell lung cancer showing that the Rb1 locus was disrupted in tumors other than retinoblastoma and osteosarcoma (reviewed in ref. 4). Since then, it has been found that most, if not all, tumors have defects in their Rb pathway through genetic lesions in the Rb1 gene itself or other genes in the pathway. The history of retinoblastoma research highlights how basic research on a rare childhood cancer can have a much broader effect on a disease that affects millions of people each year worldwide.

Although we have learned a great deal about the Rb pathway and its contribution to cancer over the past 35 years, our knowledge should be better applied to treating retinoblastoma. Despite advances in the clinical management of retinoblastoma in the United States, there are still many children with advanced bilateral retinoblastoma that lose one or both of their eyes. In developing countries, 40% to 50% of children still lose their life to metastatic retinoblastoma.

Clinical Management of Retinoblastoma

Retinoblastoma is virtually always fatal if left untreated due to tumor metastasis. The objective of retinoblastoma treatment is to save vision without risking the child’s life. Children with unilateral retinoblastoma typically undergo surgical enucleation, whereas children with bilateral retinoblastoma often receive a combination of chemotherapy and focal therapy (5). Today the focal therapies that are most widely used include laser therapy, cryotherapy, and brachytherapy (5). Every effort is made to avoid or delay radiation therapy because of the side effects associated with irradiating the surrounding tissue. In particular, children with Rb1 mutations are prone to secondary cancers later in life, and DNA damage induced by ionizing radiation accelerates this process.

One of the limitations with current clinical management of retinoblastoma is that broad spectrum chemotherapy is given systemically. This leads to significant dose-limiting side effects that can be costly and difficult to manage. Current efforts are focused on local delivery of chemotherapy, and early studies using subconjunctival carboplatin have proved effective in the clinic (6). Ideally, local delivery of targeted chemotherapy may provide improved tumor response with minimal toxicity. The recent development of animal models and identification of molecular targets for chemotherapy have accelerated progress in this area.

The First Knockout Mouse Models of Retinoblastoma

One of the reasons that research on retinoblastoma and Rb pathway has not had a greater effect on retinoblastoma treatment has been the lack of preclinical models that faithfully recapitulate genetic and histopathologic features of childhood retinoblastoma. In 1992, three groups published papers describing the phenotype of mice with a targeted deletion in their Rb1 gene (reviewed in ref. 4). Unlike children who inherit a defective copy of the Rb1 gene, heterozygous mice failed to develop retinoblastoma. Chimeric mouse studies carried out by Berns and colleagues several years later showed that p107 suppressed retinoblastoma formation in mice (reviewed in ref. 4). In 2004, three research groups generated the first knockout mouse models of retinoblastoma by conditionally inactivating Rb1 (ref. 7–9). Inactivation of Rb and p130 also led to retinoblastoma (7) showing for the first time that p130 can suppress retinoblastoma in mice.

Interestingly, a previous attempt to generate a knockout mouse model of retinoblastoma in Irbp-Cre;Rb1Δlox/Δlox,p107Δlox/Δlox;p130Δlox/Δlox mice was unsuccessful (10). The fundamental difference between Irbp-Cre and the other Cre transgenic lines mentioned above is the timing of Cre expression during retinal development. Irbp-Cre is expressed...
in cells committed to become photoreceptors just as they exit the cell cycle. In contrast, Nestin-Cre, Pax6-Cre, and Chx10-Cre are all expressed in retinal progenitor cells during development, suggesting that a retinal progenitor cell or a newly postmitotic cell distinct from the photoreceptor lineage is the cell of origin for retinoblastoma (discussed in ref. 11). Additional evidence for the identification of retinal progenitor cell as the cell of origin for retinoblastoma is the expression of retinal progenitor cell specific markers in the tumors (12) and the strong bias toward deregulated proliferation of Rbp107-deficient retinal progenitor cells compared with the newly postmitotic cells (12). These results highlight the importance of combining studies on pediatric cancer with studies on the normal development of that tissue.

### The Role of the Rb Family in Retinal Development

In addition to the general agreement that both Rb and p107 or Rb and p130 must be inactivated for retinoblastoma formation in mice, there is similar agreement on the developmental roles for the Rb family in the retina. Rb deletion in the developing retina leads to inappropriate proliferation, increased apoptosis, and differentiation defects including loss of rod photoreceptors (7, 9, 12–14). Rod photoreceptor loss has been observed in every study in which Rb is inactivated in retinal progenitor cells, including Nestin-Cre;RbLox/Lox, Pax6-Cre;RbLox/Lox, Chx10-Cre;RbLox/Lox, Max-Cre;RbLox/Lox, and Rb-deficient retinal explants (7, 9, 12–14). Similarly, acute inactivation of Rb in Rblox/lox retinal progenitor cells using replication incompetent retroviruses and square-wave electroporation has provided similar results and has shown that defective rod development is cell autonomous (12, 14). The only model that failed to show rod photoreceptor loss was IRBP-Cre;Rblox/lox retinas. This suggests that shortly after postmitotic cells have committed to the rod fate, they no longer require Rb and can develop normally. Consistent with this hypothesis, in vivo lineage analysis in Rblox/lox newborn pups using a replication incompetent retrovirus that expresses Cre (LIA-Cre) showed that some clones contained normal rods, and this proportion was increased in pups ages 12 to 36 h (14).

In both Pax6-Cre;Rblox/lox and Chx10-Cre;Rblox/lox retinas, normal rods fail to form and cells in the outer nuclear layer (ONL) express retinal progenitor cell markers and exhibit morphologic features of retinal progenitor cells in electron micrographs (12, 13). Photoreceptors are exceedingly sensitive to changes in their local microenvironment such that the cell autonomous requirement for Rb in rod maturation leads to noncell autonomous ONL degeneration in the developing retina, and this varies depending on the extent of Rb inactivation in the ONL in a particular retina. Indeed, there is some increased cell death during early rod development and several days to weeks after the normal period of rod maturation, degeneration in the ONL is observed in Nestin-Cre;Rblox/lox, Pax6-Cre;Rblox/lox, and Chx10-Cre;Rblox/lox mice (7, 9, 12, 14). Studies using a point mutant knock-in of Rb have shown that E2F1 and E2F3 are likely candidates for regulating the rod-specific genes (15) and preliminary studies have shown that simultaneous inactivation of Rb and E2F1 rescues the rod developmental defect.

In addition to defective rod development, Pax-6 Cre;Rbloplox/lox retinas exhibit failure to exit the cell cycle despite the expression of cell type–specific differentiation markers (9). Rbloplox/lox retinal cells ultimately exited the cell cycle or underwent cell death (7, 9, 12, 14). It is not yet known if loss of cell types, such as bipolar and ganglion cells in the developing Rb-deficient retina, is due to cell autonomous effects, noncell autonomous effects, or a combination of the two.

### Distinct Roles for p107 and p130 in Murine Retinoblastoma

The Rb family of proteins is made up of three members (Rb, p107, and p130) that share some functional protein domains and have overlapping roles in regulating growth control during development (16). With additional mutation of p107 or p130, levels of developmental proliferation and apoptosis increase and animals become retinoblastoma prone (Fig. 1; refs. 7–9, 12, 17, 18). Although these studies have revealed similarities in the effects of Rb deletion together with p130 or p107 loss, there are also clear differences. Retinal disorganization upon Rb and p107 mutation starts at embryonic stages, whereas disorganization occurs later, at postnatal stages in the absence of Rb and p130. This is consistent with differences in the expression of p107 and p130 throughout development, as p107 is more highly expressed at embryonic stages, whereas p130 exhibits increased expression at postnatal stages of retinal development (12). Chx10-Cre;Rblox/lox, p130loplox/lox, and Pax6-Cre;Rblox/lox;p130loplox/lox mice developed rapid bilateral retinoblastoma with 100% penetrance (18), whereas Chx10-Cre;Rblox/lox;p107loplox/lox and Pax6-Cre;Rblox/lox;p107loplox/lox animals developed predominantly unilateral retinoblastoma with partial penetrance and delayed onset of tumorigenesis. Tumor progression, with invasion of the optical nerve, lymph nodes, and brain also occurred more rapidly in Pax6-Cre;Rblox/lox;p130loplox/lox animals compared with Pax6-Cre;Rblox/lox;p107loplox/lox mice. These data suggest that p130 is a stronger suppressor of retinoblastoma than p107, and the Pax6-Cre;Rblox/lox;p130loplox/lox may be particularly useful as a preclinical model for advanced bilateral retinoblastoma which presents the greatest challenge clinically.

A close examination of tumor emergence in the Pax6-Cre;Rblox/lox;p130loplox/lox model revealed that early lesions histologically resembling retinoblastoma at P21 and P31 were located at the far retina periphery (18). The peripheral tumor localization may reflect a specific cell of origin in this region, an aspect of the regional environment permissive for tumor cell survival and/or proliferation, or a property of Pax6-Cre expression. Tumors emergence was not restricted to the peripheral retina in the Chx10-Cre;Rblox/lox;p130loplox/lox mice. Although it is possible that the tumors result from a normally postmitotic cell in this region, heterogeneity in the resulting tumors suggests that a multipotent cell type may be responsible. A critical area for future work will be to better define the nature of the cells giving rise to retinoblastoma and the similarities and differences between human retinoblastoma and the mouse models.

### Species-Specific Differences in Retinoblastoma Susceptibility

It has been well established that inactivation of the RB1 gene is the initiating genetic lesion for retinoblastoma. However, as mentioned above, Rb-deficient mouse retinoblasts do not form retinoblastoma. Detailed characterization of the expression of the

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4 M.A. Dyer, unpublished observations.
Rb family (Rb, p107, and p130) in the developing mouse and human retina and analysis of intrinsic genetic compensation in Rb-deficient mouse and human retinoblasts has suggested a model to account for this species-specific difference in retinoblastoma susceptibility (12). In the developing mouse retina, p107 is the major family member expressed in embryonic retinal progenitor cells and Rb is the major family member expressed in postnatal retinal progenitor cells (12). There is some overlapping expression between Rb and p130 in the postnatal and adult mouse retina. When Rb is inactivated in the developing mouse retina, p107 is increased in a compensatory manner in many cells (12, 14). We hypothesize that the inactivation of Rb is not sufficient for retinoblastoma formation in the mouse due to intrinsic genetic compensation by p107 or intrinsic genetic redundancy by p130. As a result, retinoblastoma only forms in Rb;p107-deficient or Rb;p130-deficient retinoblasts in the mouse retina. In the developing human retina, RB1 is the major family member expressed. When RB1 is inactivated, there is little, if any, compensation by p107 or p130. This may explain why inactivation of RB1 is sufficient for retinoblastoma formation in humans but not in mice.

Refinement of this model will require a fuller understanding of the specific retinoblastoma-originating cells and the response of such cells to Rb deletion. Whereas p107 increases occur in postnatal stages of murine retinal development after Rb deletion, this is not seen at embryonic stages (7, 12). In embryonic retinas, the levels of a critical regulator of p107 activity, cyclin D1, decreases in response to Rb deletion, which may also restrain the effects of Rb activity at the level of pocket protein activity. The developmental stage at which the cells in the retina are prone to tumor initiation upon Rb family mutation is not known. Although these studies clearly show that the genetics of Rb family inactivation is different in humans and mice with respect to retinoblastoma susceptibility, there is a common theme across species. In both mice and humans, Rb family function must be inactivated to initiate retinoblastoma; therefore, the preclinical models recapitulate these early events in retinoblastoma tumorigenesis.

**Secondary Genetic Lesions in Retinoblastoma and Targeted Chemotherapy**

Whereas it has been well established that RB1 inactivation is the initiating genetic lesion in retinoblastoma, only a handful of secondary genetic lesions have been carefully analyzed. The newly developed knockout mouse models of retinoblastoma provided a critical tool to begin to identify secondary genetic lesions in retinoblastoma. Using the Pax6-Cre;RbLox/Lox;p107−/− and Pax6-Cre;RbLox/Lox;p130−/− models, it was recently found that N-myc also undergoes gene amplification in murine retinoblastoma (18). The N-myc oncogene has also been found to undergo gene amplification in a subset of human retinoblastomas. The frequency of

**Fig. 1.** Kinetics of murine retinoblastoma development. Plotted is the time to the first observation of unilateral and bilateral retinoblastoma in Pax6-Cre;RbLox/Lox with additional deletion of p107 or p130. Inset, Pax6-Cre;RbLox/Lox;p107−/− mouse at age of 18 wks with bilateral retinoblastoma.
N-myc amplification (found in 5 of 61 tumors overall) is similar to the frequency in human retinoblastomas (18). In tumors lacking Rb and p130, N-myc amplification was associated with metastasis, suggestive of a role for N-myc in tumor progression. Common amplification of N-myc in murine and human retinoblastoma provides further evidence that the murine models faithfully recapitulate human disease.

It was also recently shown that Chx10-Cre;Rb+/−/p107−−/−; p53+/−/− mice develop bilateral invasive aggressive retinoblastoma with 100% penetrance. This is in contrast to Chx10-Cre;Rb+/−/−; p107−−/− mice that develop unilateral minimally invasive retinoblastoma with ~50% penetrance. These data suggested that p53 suppressed retinoblastoma in mice. However, previous studies have shown that the p53 gene was intact in human retinoblastomas. Molecular genetic analyses revealed that the MDMX gene was amplified in 65% of retinoblastoma cases and MDM2 was amplified in an additional 10% (19). Using mouse models of retinoblastoma, cultured retinoblastoma cell lines, human fetal retina, and primary retinoblastomas from enucleated eyes, it was confirmed that amplification of MDMX suppresses p53-mediated cell death in Rb1-deficient retinoblasts and promotes clonal expansion of the tumors. Whereas these findings do not rule out the possibility of other nonapoptosis-related functions of p53 inactivation in tumorigenesis, there is a clear connection between suppression of p53-mediated apoptosis and retinoblastoma progression.

Not only do these data argue that retinoblastoma does not arise from an intrinsically death resistant cell as previously proposed (9), but it provided the first specific target for chemotherapy. Nutlin-3, a small molecule inhibitor of MDM2 was found to bind to MDMX and prevent it from binding to p53. Using some of the newly developed preclinical models of retinoblastoma (20), Nutlin-3 was found to have potent antitumor effects, especially when combined with another drug that also induces a p53 response, topotecan (19). This is the first example of a locally delivered targeted chemotherapy for any pediatric cancer. Importantly, preclinical models were essential for these studies on the basic biology of retinoblastoma and for testing new drug combinations and methods of delivery.

**Summary**

The past three years have witnessed a remarkable advancement in our understanding of retinoblastoma biology and the development of new therapeutic interventions. The field is rapidly moving toward implementing locally delivered targeted therapies which may improve outcomes while reducing toxicity associated with radiotherapy or systemic broad-spectrum chemotherapy. These advances have been built on the strong foundation of genetic studies of retinoblastoma over the past several decades and have been accelerated by the development of new mouse models of retinoblastoma. It is encouraging that there is broad agreement from independent laboratories using different approaches about the role of Rb family in retinal development and retinoblastoma. More importantly, these studies have raised exciting new questions that will advance our understanding of tumorigenesis in the developing central nervous system and the role or tumor suppressor genes in normal developmental processes in the years to come.

**Acknowledgments**


**References**

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