Review Article

Transgenic Models in Retinoblastoma Research

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Key WordsRetinoblastoma · Transgenic models · Tumorigenesis · LH β -Tag · Knockout models**Abstract**

Understanding the mechanism of retinoblastoma (Rb) tumor initiation, development, progression and metastasis in vivo mandates the use of animal models that mimic this intraocular tumor in its genetic, anatomic, histologic and ultrastructural features. An early setback for developing mouse Rb models was that Rb mutations did not cause tumorigenesis in murine retinas. Subsequently, the discovery that the p107 protein takes over the role of pRb in mice led to the development of several animal models that phenotypically and histologically resemble the human form. This paper summarizes the transgenic models that have been developed over the last three decades.

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LH β -Tag Mouse Model

Windle et al. [1] developed the first retinoblastoma (Rb) transgenic mouse model in 1990 using the simian virus 40 (SV40) large T antigen (Tag) gene cohybridized with the luteinizing hormone β -subunit (LH β) gene as promoter. This ensured a low expression of the Tag gene specifically in the gonadotropic cells of the anterior pituitary in many murine lines, one of which developed heritable bilateral ocular neoplasms at about 5 months [1]. The SV40 genome has several oncogenes in both the large T and the small t region which are predicted to cause a neoplastic transformation of the tissues by binding to p53, the pRB family and phosphatase pp2A proteins [2]. The murine eye tumors showed the histological characteristics of human Rb, including the presence of the neuronal marker NSE and synaptophysin

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and the absence of S-100 and vimentin [3]. The tumors also showed an endophytic and exophytic growth with invasion of the retina, choroid, optic nerve and vitreous seeding. This model was followed by the development of interphotoreceptor retinoid-binding protein promoter-based Tag Rb transgenic mice which developed tumors that also showed the neuronal characteristics of human Rb [4, 5]. The ocular tumors in these mice exhibited both Flexner-Wintersteiner and Homer-Wright rosettes, which can be observed using light and electron microscopy when the mice are 5 months of age. However, the question regarding the nature of the cell of origin still remains unanswered and debated with histologic evidence pointing towards the amacrine cell layer [6].

The early stages of tumor development in the Tag model were investigated by Pajovic et al. [7] by tracking Tag protein expression in the retina. The SV40 Tag causes neoplastic transformation of the developing retinal cells. Their studies revealed that Tag expression starts within the nuclei of the inner nuclear layer by postnatal day 8 (P8), increases by P21 and then shows a steady decline until the P28 stage. A decreased expression was observed along with an increased expression of activated caspase-3, which indicates apoptosis. The authors also reported expression of Müller glial markers in these Tag-positive cells, which led to the conclusion that the cell of origin in these tumors might be a subset of progenitor-like Müller glial cells that undergoes transformation upon Tag expression.

To further characterize the tumor-initiating cells of the Tag model, Wadhwa et al. [8] established a murine Rb cell line using tumors from P7 mice of the Pax6 Tag model. They identified the presence of a subset of tumor-initiating cells that express known stem cell markers such as CD133, Nestin and Sox2. These CD133⁺ cells were able to generate neurospheres in vitro and to form transplantable tumors in vivo which were identical to the parent tumor. The transgenic Rb Tag model has been widely used over the past two decades for studying Rb tumorigenesis and also for evaluating therapeutics such as local chemotherapy, radiation therapy, vascular targeting therapies and cryotherapy [9–13].

Conventional therapies such as local carboplatin chemotherapy and external beam radiotherapy along with angiogenesis inhibitors like anecortave acetate caused increased apoptotic cell death in the Tag Rb model [9]. Intraocular tumor growth was observed to be inhibited by subconjunctival delivery of carboplatin in 5-week-old transgenic BLH SV40 Tag-positive mice with Rb by Murray et al. [10]. Cryotherapy successfully limited tumor growth in these transgenic mice. Combination therapy using intravitreal carboplatin and external beam radiotherapy resulted in better tumor growth control [11]. The use of optical coherence tomography to assess tumor growth in vivo and quantify its development from the early tumor initiation to the advanced stage was also carried out successfully in this model [14, 15].

The Rb Tag transgenic model has been an invaluable tool for understanding the origin and tumorigenesis of Rb and also for assessing various therapeutic interventions at a preclinical stage. However, the use of viral oncogenes to induce tumorigenesis has its setbacks as the interactions of the oncoproteins with other tissue proteins are not fully understood. Also, the Tag model does not exhibit the focal and clonal tumors which are characteristic of human Rb. These two striking disadvantages warrant the need for developing a conditional knockout (KO) model that mimics both the origin and development of human Rb.

Rb KO Animal Models

The early nineties witnessed the development of gene knockout technology that led to the development of several disease models and other transgenic models that lacked spatial expression of specific genes. Based on this, several researchers attempted to generate Rb

chimeric mouse models by conditional knockout of the Rb gene in the retinas. However, these animals did not develop ocular tumors even though the Rb^{-/-} cells contributed to the developing central nervous system [16, 17]. An interesting observation was the presence of ectopic mitoses and massive cellular degeneration in the developing retina of these chimeric embryos. Robanus-Maandag et al. [18] identified p107 as the factor preventing Rb in Rb double KO (DKO) mice. The Rb-related gene p107 was found to be required to facilitate the oncogenic program following Rb inactivation and to function as a tumor suppressor. The protein p107 is highly similar to pRB1 with respect to its sequence and biologic function [19]. MacPherson et al. [20] and Donovan et al. [21] showed that postnatal murine retinas lack Rb and that their explant cultures showed a marked upregulation of the p107 gene, unlike human retinas. This confirmed the hypothesis that p107 plays a role similar to that of pRB and compensates for the protein in its absence. The embryos which were double knockouts for Rb and p107 exhibited high developmental lethality. In order to generate living and breedable Rb mice, selective knockout of these genes in the developing retina was attempted using Cre-lox technology [22]. However, it was observed that the loss of Rb, p107 or p53 in photoreceptors did not result in any malignant transformation. Further attempts were carried out using nestin, Chx10 and Pax6 promoters to create Rb KO models by conditional gene deletion in retinal progenitor and other cells [20, 23–25]. The models thus generated took about 3–9 months to develop detectable tumor masses with tumor cells expressing progenitor, amacrine and Müller glial markers. Nestin-Cre and Pax6-Cre could not be used for further preclinical studies due to their late onset of tumor growth, low penetrance and nonautonomous cellular effects. It was observed that all the viable nestin-Cre KO mice (n = 5/11) developed tumors in the eye with 4 having bilateral tumors histologically similar to the human form with signs of invasion into the surrounding muscle. The authors reported that the tumors were mostly found filling the area behind the lens and in the inner nuclear layer. Homer-Wright rosettes were observed, and the tumor foci exhibited high levels of mitoses and apoptosis [20]. Phenotypic features that were shared between the Rb deletion models and Cre transgenic lines were the high numbers of mitotic figures, increased cell death in the inner retinal layer from day E16.5–18.5 onwards [17] and photoreceptor degeneration [18, 20], with the most conspicuous effect being the extension of the retinogenesis period [20, 24, 25] and the ectopic proliferation in the retinal ganglion cell layer [26]. The addition of another mutation of p107 in these animal models led to an increase of many developmental phenotypes associated with retinal Rb loss, which also threw light on the functional synergy between these family members [18, 20, 24]. Apoptotic cell death was observed in specific cell layers following Rb deletion, and in α -Cre Rblox/lox mice, the rod, bipolar and ganglion cells were completely degenerated [24]. In the Chx10Cre Rblox/lox model, the bipolar cells did not undergo apoptosis. These experiments showed diverse phenotypic and biological differences between the various models, which could be due to the genetic heterogeneity of the mouse strains and differences in the temporal and spatial expression of the Cre gene across the cell types [27]. These models have rendered an insight into the possible pathway of Rb tumor initiation following pRB loss and progression, which makes them suited for testing novel pathway-directed therapies.

The Rb/p107 DKO models demonstrated high levels of retinal proliferation as well as apoptosis, although many of the horizontal, amacrine and Müller cells survived the Rb and p107 mutation [18, 25, 28, 29]. Many of the DKO animals subsequently developed Rb, and it was interesting to note that, since the tumors arose from highly apoptosis-sensitive cells, the cell of origin seems to bear an inherent resistance to cell death cues. Human Rb are also known to exhibit areas with increased apoptosis. The delayed tumorigenesis and incomplete penetrance observed in this model led to the development of the Rb/p130 DKO model. Rb-like protein 2, commonly known as p130, is known to play an important role in retinal devel-

Fig. 1. Transgenic animal models in Rb.

Table 1. Studies using transgenic mouse models to study Rb tumorigenesis, invasion and therapeutic evaluation

Rb transgenic model	Tumor initiation and progression	Metastasis	Assessment of therapies
Tag model	[1, 3, 8]	[1, 3, 6]	[9–13, 37–47]
Rb ^{-/-} p107 ^{-/-}	[18, 20, 25]	[25]	–
Rb ^{-/-} p130 ^{-/-}	[20, 30]	[20]	–
α-Cre Rblox/lox p130 ^{-/-}	[25, 31]	[25]	–
NesCre1 Rblox/lox p130 ^{-/-}	[20, 25]	[20, 25]	–
Chx10-Cre;RbLox/Lox;p107 ^{-/-}	[23, 24]	–	–
Chx10-Cre;RbLox/Lox;p130 ^{-/-} ;p107 ^{+/-}	[23, 31]	[31]	–
Chx10-Cre;RbLox/Lox;p107 ^{-/-} ;p53Lox/Lox	[23, 29]	[23, 29, 35]	[35, 36]

opment, cell cycle regulation and tumor suppression. NesCre1 Rblox/lox p130^{-/-}, α-Cre Rblox/lox p130^{-/-} and Rb^{-/-}p130^{-/-} chimeras substantiated the evidence towards the role of p130 in Rb development [20, 25, 30]. However, the histological deformities in the embryonic retinal development as seen in the NesCre1 Rb/p107 DKO retinas were absent in these models [20]. All of the chimeric mice that lack both Rb and p130 (NesCre1 Rblox/lox p130^{-/-} and α-Cre Rblox/lox p130^{-/-}) form Rb with a wide array of expressed markers, which include those identifying amacrine and horizontal cell genes [20, 25, 30]. Of these models, the α-Cre Rblox/lox p130^{-/-} chimeras are more suitable for studying advanced Rb owing to their rapid tumor progression and metastasis. They present with lesions consistent with Homer-Wright rosettes at the retinal periphery as early as P21–31 [25]. The tumor cells proliferate to fill the

posterior chamber and were observed to invade the optic nerve and extend to the brain. Both DKO models, Rb/p130 and Rb/107, have features that bear resemblance to human Rb with neuroblastic differentiation and have similar histologic features. Ajioka et al. [31] have generated transgenic mice that express a single copy of Rb1, p107 and p130 in the developing retina. This study helped reveal the role of the 3 Rb family members in Rb formation. In the heterozygous p107 mutant retina, it was observed that the horizontal neurons start to differentiate normally, but after several weeks, they resume the cell cycle, expand clonally and produce bilateral Rb that invade into the bone marrow. It is interesting to note that the horizontal cells of the other KO mice [Rb(+/-);p107(-/-);p130(-/-) or Rb(-/-);p107(-/-);p130(+/-)] were not affected, which indicates that a single copy of Rb or p130 is enough to control the horizontal cell expansion. The study showed for the first time that neurons were capable of re-entering the cell cycle after differentiation and can form invasive tumors, hinting that Rb originates from dedifferentiation of horizontal cells [31]. McEvoy et al. [32] conducted an extensive study using the Chx10-Cre system on six mouse lines and generated the KO mice that were histologically similar to each other and to human Rb. The markers expressed in these tumors point to amacrine cell differentiation. The rate of tumor progression and penetrance varied significantly in these strains, which led to their subsequent study on the genetic-epigenetic changes that occur during tumorigenesis [33]. A study by Laurie et al. [34] showed that several foci of clonal retinal tumors were formed in newborn p53-deficient mice that had a replication-incompetent retrovirus encoding the E1A 13S oncogene. The model was used to test the efficacy of topotecan in combination with carboplatin and vincristine for Rb treatment. This model did not exhibit any signs of metastasis during the experimental period, but after 8 months it showed some invasion into the optic nerve and anterior chamber, thereby limiting its use as a metastatic Rb model.

Transgenic Models in Preclinical Testing

The transgenic Rb models are well suited for preclinical testing owing to their known genetic lesion and spontaneous tumor development in the eye with well-established vascularization. The genetic KO models serve best for this purpose, as they do not only create lesions that are identical to human Rb but also share its initial mutations that lead to tumorigenesis.

These models validate the assumption that an *in vivo* tumor model that comprises the genetic signature of the human malignancy is capable of replicating clinical behavior. Assessing combination therapies *in vivo* has been a challenge for clinical trials, which is being significantly addressed by these animal models [10, 11, 34–36]. One of the few promising preclinical studies used systemic topotecan and subconjunctival carboplatin for Rb treatment, which not only was shown to ablate the tumor in the KO mice (Chx10-Cre;RbLox/Lox;p107-/-;p53Lox/Lox) but even restored vision in some of the long-term survivors [35]. The LHβ-Tag and p53 triple-knockout (TKO) models are currently the most preferred transgenic Rb models for preclinical testing of existing and novel therapeutic regimens (table 1).

In conclusion, several genetic models of Rb have been established that have helped to understand Rb tumorigenesis and metastasis. These models are more reliable when compared to the orthotopic models for studying the biokinetics of tumor formation, the role of the tumor microenvironment and invasion as well as for assessing several therapeutic strategies for the treatment of Rb (fig. 1). Genetic animal models form the bridge between translating the basic knowledge about the pathogenesis of Rb and the development of successful therapies.

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