Effects of an $Rb$ mutation in the mouse

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The retinoblastoma gene is mutated in several types of human cancer and is the best characterized of the tumour-suppressor genes. A mouse strain has been constructed in which one allele of $Rb$ is disrupted. These heterozygous animals are not predisposed to retinoblastoma, but some display pituitary tumours arising from cells in which the wild-type $Rb$ allele is absent. Embryos homozygous for the mutation die between days 14 and 15 of gestation, exhibiting neuronal cell death and defective erythropoiesis.

As a means of addressing these and related questions, we have undertaken the construction of a mouse strain in which one allele of the $Rb$ gene has been disrupted. This effort has been made possible by the development of methods for gene targeting in mouse embryonic stem (ES) cells. In addition to potentially serving as a model for human familial retinoblastoma (and other human familial cancer syndromes caused by inheritance of a mutant tumour suppressor gene), mice that are heterozygous for an $Rb$ mutation can be interbred to create homozygous animals or embryos. An analysis of the homozygous mutant phenotype should help define the function of $Rb$ in normal cells and provide a basis for understanding the consequences of $Rb$ mutation in tumorigenesis. We report here on the phenotypes of such $Rb^{-}$ heterozygous and homozygous mice.

$Rb$ gene targeting

We have used the positive-negative selection method of Mansour et al. to disrupt one allele of $Rb$ in mouse ES cells. A region of the murine $Rb$ gene containing exons 3 and 4 was modified as shown in Fig. 1. A bacterial neo gene (neo) expression cassette was inserted into the third intron of the gene in place of a roughly 900 nucleotide (nt) fragment of the intron; the direction of transcription of neo is opposite to that of $Rb$. In addition, three nucleotide changes were introduced into exon 3 by site-directed mutagenesis, creating two termination codons and a new PsI restriction site within the exon. The herpes simplex virus thymidine kinase (HSV-tk) gene was ligated upstream of the $Rb$ sequences, in order to allow for counter-selection against ES cells that acquired the targeting vector by random integration of the entire DNA construct.

After introduction of the targeting vector into D3 ES cells (derived from strain 129/oiv), the resulting neo-positive (G418-resistant), HSV-tk-negative (gancyclovir-resistant) clones were screened by Southern blotting. Probes located in introns 2 and 3 detect a 10 kilobase (kb) PsI fragment from wild-type DNA and a mutant-specific fragment of about 5 kb (Fig. 1). Of 150 ES clones screened, five had acquired the exogenous $Rb$ sequences by homologous recombination and were, therefore, heterozygous for the mutation (not shown); the HSV-tk counter-selection resulted in a roughly 10-fold reduction in the number of ES cell colonies. The presence of the point mutations in the third exon was further verified by DNA sequencing of a region of DNA amplified by polymerase chain reaction (PCR) (see Fig. 1; data not shown). Translational termination at the stop codons in exon 3 would result in the synthesis of an N-terminal pRb peptide of $M_{r} \approx 10$K; full-length mouse pRb has a mass of 105K (ref. 29).

Chimaeric animals were created by injection of heterozygous ES cells into C57BL/6 blastocyst-stage embryos, and contribu-
Fig. 1 Disruption of Rb in ES cells. The Rb-targeting vector pRBX3T-KO was constructed by inserting fragments of the mouse Rb gene extending from intron 2 to intron 4 (isolated from a strain 129/Sv germ line library) into the plasmid pPNT12. The regions of homology consist of fragments of 1.1 kb (BamHI-B) to Asp718 (A) and 8.5 kb (Asp718 to Mbol, D). The paga-neo expression cassette is located ~200 nt 3′ to Rb exon 3 in place of a 900 nt Asp718 fragment in intron 3.

Three base changes were introduced into exon 3, creating two termination codons and a new PstI recognition site. The wild-type and mutant sequences are shown; the altered exon 3 is abbreviated 3′. The HSV-tk gene (also under the control of the pAg promoter) is located adjacent to the Rb intron 2 sequences. The HSV-tk and pAg genes are under transcriptional orientation opposite to Rb. Homologous recombination between the targeting vector and one chromosomal Rb allele produces a mutant Rb allele (mut) that can be distinguished from wild-type (wt) Rb by Southern blotting of PstI-digested genomic DNA using the probes [A] and [B], which are located on each side of the sites of recombination. The DNA of heterozygous ES cells would contain a wild-type fragment of 10.6 kb and mutant-specific fragments of ~5 kb. The two alleles can also be distinguished by PCR using primer pairs that specifically amplify wild-type (RK3 and RK3) or mutant sequences (RK3 and pAgK3).

Methods. Electroporation of pRBX3T-KO into D3 ES cells and the subsequent drug selection was done using the conditions described by Tybulewicz et al., except that primary mouse embryonic fibroblasts were used as feeder cells. Genomic DNA was isolated from ES cell clones using the method of Laird et al. and screened by Southern blotting of ~10 μg PstI-digested DNA. Samples were separated in 0.9% agarose gels, transferred to nylon membrane (Hybond-N, Amersham) and hybridized as described. Blots were initially probed using probe A (which is not contained within the targeting vector) and candidate heterozygous clones rescanned using probe B. The presence of the exons 3 mutations was confirmed by DNA sequencing (Sequenase 2.0, USB) of DNA amplified by PCR (AmpliTaq, Perkin-Elmer-Cetus) using primers RK3 and pAgK3. Primer sequences: RK3 (5′-AATTTGGGCGGCCATTCTGCATTCTTATGCG-3′), RK3 (5′-CCATGTTCCGTTCCCTGAG-3′), pAgK3 (5′-GAGAAGCGAGATCGACG-3′).

Tumours in Rb<sup>−/−</sup> heterozygotes

Humans heterozygous for a deleterious mutation in Rb have a roughly 90% likelihood of developing retinoblastoma by the age of three. In contrast, of more than 100 heterozygous mice that have been followed for up to 11 months of age, not one has developed retinoblastoma. In addition to superficial examination of all heterozygotes, 20 of these animals were screened by indirect ophthalmoscopy, and an additional six eyes were examined by histological sectioning. No signs of either retinoblastoma or precursor lesions (that is, retinomas) were evident.

In addition to their strong predisposition to retinal tumours in early childhood, human carriers of an Rb mutation have an increased risk of developing osteosarcoma and other tumours later in life. For this reason, we monitored the germ line heterozygotes, as well as the chimaeras made from heterozygous ES cells, for the development of other tumour types. Indeed, we have recently observed the first indication of any cancer predisposition in these animals. Four chimaeras and five germ line heterozygotes were killed after displaying severe wasting between the ages of 8 and 10 months. Autopsy revealed large tumours (~6 mm in diameter) in the area of the pituitary gland in all nine animals. These tumours have been classified histopathologically as adenocarcinoma of the pituitary; a section of one tumour is shown in Fig. 3a. The five pituitary tumours observed in heterozygotes arose in a population of the 20 animals that were at least 9 months old. Southern blot analysis shows an absence of the wild-type allele of Rb and retention of the mutant allele in the DNA of eight tumours tested (Fig. 3b; data not shown). This result confirms the role of Rb in the genesis of these tumours, and is consistent with the "two-hit" model of Rb mutation of the gene first proposed by Knudson.

The homozygous phenotype

These heterozygous animals have also been useful for establishing the requirements for Rb function during mouse development.
ment. We mated male and female Rb<sup>−/−</sup> heterozygotes and determined the genotypes of the offspring at 3 weeks of age. If Rb function were dispensable for normal mouse development, we would have expected a 1:2:1 ratio of wild-type, heterozygous and homozygous mutant offspring. If, however, Rb were required for some aspect of embryogenesis, homozygous embryos would not survive to term. The genotypes of the first 64 offspring of such heterozygous crosses revealed 22 wild-type, 42 heterozygous and 0 homozygous animals. This result clearly indicates that Rb is an essential gene in the mouse.

By collecting embryos at progressively earlier times of development, we have been able to delineate the timing of the death of Rb<sup>−/−</sup> homozygous embryos. Of 70 embryos collected at 12.5 days postcoitum (d.p.c.), 13 were Rb<sup>−/−/−</sup>. Because this is within the expected ratio, we can conclude that the Rb<sup>−/−</sup> mutation does not affect embryonic viability up to day 12.5 of gestation. Furthermore, on a gross morphological level, the Rb<sup>−/−/−</sup> embryos were indistinguishable from their littermates. This viability is not explained by residual expression of Rb, as no Rb is seen in western blots of cell lysates of 12.5 d.p.c. embryos (Fig. 4). We have also shown a lack of Rb expression in Rb<sup>−/−/−</sup> primary embryo fibroblasts using indirect immunofluorescence and immunoprecipitation with a combination of three anti-Rb monoclonal antibodies which recognize different epitopes (data not shown). Because of the lack of high-affinity antibodies recognizing epitopes in the amino-terminal region of Rb, we were unable to assay for the production of the N-terminal 10K fragment of the protein that might be synthesized from the Rb<sup>−/−</sup> allele.

At day 15.5 of gestation, viable Rb<sup>+/+</sup> embryos were also present at the expected frequency (18/53), but at this age most of the homozygotes (14/18) were obviously abnormal. The 13.5 d.p.c. Rb<sup>−/−/−</sup> embryo shown in Fig. 5a exemplifies the mutant phenotype: the mutant is paler and has less obvious superficial vasculature than the normal littermate; it also has significant oedema, particularly in the pericardial space, and the liver is slightly reduced in size. The lethality of the Rb<sup>−/−</sup> mutation was first evident at 14.5 d.p.c., when three of five homozygous embryos were found dead (determined by no heartbeat). All six Rb<sup>−/−/−</sup> embryos recovered at 15.5 d.p.c. were dead.

Inhibition of hepatic erythropoiesis

Largely on the basis of the appearance of the mutant embryos at 13.5 d.p.c. (Fig. 5a), we suspected that part or all of the lethal phenotype could be explained by severe anaemia. In fact, a role for Rb in the control of erythroid development was first suggested by earlier work, which demonstrated that at 12.5 days of gestation, the liver was the site of maximal expression of Rb<sup>−/−</sup>. At this stage of development, the liver is the major erythropoietic organ<sup>14</sup>. A block in hepatic erythropoiesis in Rb<sup>−/−/−</sup> embryos could account for the apparent anaemia and might suffice to cause the observed lethality. Furthermore, the survival of these Rb<sup>−/−</sup> homozygous mutants until day 13 or 14 of gestation suggests that the primary, yolk sac-derived erythropoiesis, which occurs between days 8 and 11 of gestation<sup>14</sup>, is not dependent on Rb function.

Examination of the livers and blood of 13.5 d.p.c. mutant embryos indicated substantial disruption of erythropoiesis. Livers of wild-type 13.5 d.p.c. embryos are densely packed with cells (90% of which are in the erythroid lineage) (Fig. 5b), whereas the livers of Rb<sup>−/−/−</sup> embryos are lacy in appearance, lacking extensive cellularization. Blood smears from these animals also show evidence of a deficiency of erythrocyte production. We measured the ratio of circulating primitive erythrocytes (nucleated red cells derived in the yolk sac) to the nucleated, definitive erythrocytes born in the liver. Control embryos contain on average 43% nucleated cells at this stage (Fig. 5d), whereas Rb<sup>−/−/−</sup> embryos have on average 68% nucleated cells (Fig. 5e). (These percentages are derived from blood counts of a total of more than 2,000 cells isolated from three control and three mutant embryos.)
The observed inhibition of definitive erythropoiesis in \( Rb^{33/33} \) embryos could be due to a number of factors, including a failure of the haematopoietic stem cells to migrate efficiently to the liver, a defect in the hepatic microenvironment that normally supports erythropoiesis, or an intrinsic inability of \( Rb^+ \) erythroid progenitor cells to achieve end-stage differentiation. We have begun to examine the \( Rb \) requirement in this system by determining the in vitro differentiation capacity of erythroid precursors isolated from mutant and control embryos. Single-cell suspensions made from the livers of 12.5 d.p.c. \( Rb^{33/33} \) and control embryos were plated in vitro under conditions optimized for erythroid differentiation. Plates were seeded with equal numbers of cells, although we consistently observed two- to fivefold fewer cells in the livers of homozygous mutant embryos. Erythroid colonies were then examined on each of the first 7 days after plating.

We observed comparable numbers of small haemoglobinized (CFU-E) colonies from mutants and controls three days after plating. Thus, the failure of \( Rb^{33/33} \) embryos to produce definitive erythrocytes efficiently, is not a consequence of the absence of the relevant precursor cells in the liver. But the CFU-E colonies derived from \( Rb \) homozygotes differed qualitatively from controls, being slightly paler in colour (Fig. 6a, b). Also, control CFU-E colonies were visible up to 5 days in culture, whereas the mutant CFU-E colonies were no longer apparent at this time. Large haemoglobinized colonies from \( Rb^{33/33} \) embryos were also paler than controls (not shown), and at day 4 were three times more numerous in mutant samples than in control samples.

A number of colonies from mutant and control platings were picked and stained with Wright-Giemsa. Control CFU-E colonies contain a significant fraction of enucleated erythrocytes (Fig. 6c), whereas the mutant CFU-E colonies have very few if any of these mature red cells (Fig. 6d). Instead, the mutant colonies contain increased number of a small, very densely staining cell type that most resembles a late normoblast. Similarly, large erythroid colonies from control embryos consist of about 45% enucleated erythrocytes (Fig. 6e); mutant colonies have fewer than 5% enucleated cells (Fig. 6f). Thus, \( Rb^{33/33} \) erythroid precursors seem to be intrinsically defective in reaching end-stage differentiation. This result is consistent with the
inhibition of definitive erythropoiesis observed in vivo. Our conclusion from this analysis is that Rb<sup>-/−</sup> embryos are unable to produce sufficient numbers of mature erythrocytes beginning at about day 13 of gestation. This condition would be expected to lead to hypoxia and the eventual death of the embryo.

**Neuronal cell death**

We have histologically analysed several Rb<sup>-/−</sup> homozygous mutants for evidence of other effects of the lack of Rb expression during development. With the exception of damage to the dermis and underlying mesenchyme from oedema, most non-haematopoietic tissues appear normal until the death of the Rb<sup>-/−</sup> embryos at about 14.5 d.p.c. But serial cross-sections from day 12.5 and 13.5 embryos have shown fairly widespread neuronal cell death in mutants compared to controls. Evidence of increased cell death was observed in the spinal cord (Fig. 5g), as well as in the dorsal root ganglia and portions of the hindbrain. The cell death does not seem to be a secondary effect of hypoxia induced by anaemia, because it is evident at 12.5 d.p.c., before the manifestation of the erythropoietic defect.

In addition to necrosis there is a marked increase in number of mitotic figures in and away from the ventricular zone of the spinal cord and the hindbrain of the mutant embryos. Finally, we paid particular attention to the retinas of Rb<sup>-/−</sup> homozygous mutants for any evidence of neoplastic transformation. As shown in Fig. 5f, however, up to day 13.5 of gestation, the absence of Rb function does not seem to affect development of the eye.

**Discussion**

Familial retinoblastoma is a highly penetrant disease in humans. About 90% of carriers develop retinal tumours by the age of three<sup>3</sup>; these individuals have on average six tumour foci, often affecting both eyes. In stark contrast, the heterozygous mice reported here, which carry an analogous mutation in Rb, are not predisposed to retinoblastoma. We have considered the possibility that this species difference is due to the relatively small number of target cells in the mouse retina in which inactivation of the wild-type allele of Rb would lead to transformation. But the apparent normality of the retinas of 13.5 d.p.c. Rb<sup>-/−</sup> embryos argues that even if Rb<sup>-/−</sup> retinoblasts were produced in heterozygous mice, they would not be transformed.

Retinoblastoma has, nonetheless, been observed in mice<sup>46</sup>. A transgenic strain expressing SV40 T-antigen in the retina develops this tumour at a high frequency. In light of our results, we believe that transformation in this system involves functions of T-antigen beyond its effects on p53<sup>36</sup>, including, for example, sequestration of the p53<sup>36</sup> protein<sup>36</sup>. Therefore, the failure to observe retinoblastoma in Rb<sup>-/−</sup>-heterozygous mice may be explained by a requirement for mutations in additional genes. Spontaneous retinoblastoma has not been observed in any species besides humans, perhaps suggesting that human retinoblasts are uniquely sensitive to the simple loss of Rb function.

Although mice that are heterozygous for an Rb mutation do not develop retinoblastoma, they are predisposed to malignancy. All nine pituitary tumours described here arose from cells that were heterozygous for the Rb<sup>-/−</sup> mutation and eight were shown to have lost the wild-type allele of the gene. This is a strong indication of Rb involvement in pituitary tumorigenesis in these cases and is the first example of Rb mutation in any primary mouse tumour. It is not known whether Rb is mutated in human pituitary tumours.

We have also established that Rb is an essential gene in the mouse. Homozygous mutant embryos die between days 13.5 and 15.5 of gestation, apparently from a failure to produce definitive erythrocytes efficiently in the liver. Given the known association of Rb with the transcription factors c-Myc, N-Myc and E2F<sup>35,36</sup>, this developmental defect might be explained by the ability of Rb to associate with and potentiate the activity of transcription factors that are essential for erythrocyte-specific gene expression. The haematopoietic phenotype of embryonic embryos deficient for c-myc is very similar to that described here<sup>35</sup>, suggesting a possible functional connection between c-Myc and Rb.

An alternative model, more in keeping with the putative role of Rb as a cell-cycle regulator, would state that Rb acts to cause a cessation of cell division which is a prerequisite to terminal differentiation. Accordingly, the absence of Rb may trap precursor cells of particular lineages in a proliferative compartment and preclude entry into end-stage differentiation. Indeed, there is evidence of increased mitotic activity in the developing nervous system of the Rb<sup>-/−</sup> mutant embryos, although it is not known whether there is also increased proliferation of haematopoietic precursors in these animals. To explain the observed reduction in cell number in these lineages, it is necessary to argue that the inability to terminally differentiate ultimately leads to cell death. It is also possible that the observed increase in mitotic activity in the nervous system occurs in response to increased cellular turnover.

We have been struck by the apparent dispensability of Rb function for much of mouse development. It would seem that Rb does not play a critical role regulating cell death or differentiation up to day 13-14 of gestation. Alternatively, in the absence of Rb expression, an Rb-related gene, such as p107 (ref. 40), might provide an analogous function in most tissues.
The Rb-homozygous embryos offer advantages beyond the insights into regulation of erythropoietic and neuronal lineages described here. Study of the role of Rb inactivation in tumour progression has been confounded by the multitude of other genetic lesions that are invariably present in tumour-cell genomes. The Rb\(^{+3/4}\) homozygous embryos now provide a source of a variety of cell types that, although lacking Rb function, would seem to be genetically otherwise intact. Such cells should prove invaluable in dissecting out the unique role that this gene plays in regulation of tumour cell proliferation.

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**LETTERS TO NATURE**

X-ray detection of the eclipsing millisecond pulsar PSR1957+20

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In the binary millisecond pulsar system PSR1957+20 (ref 1, a wind from the pulsar is ablating a low-mass (0.02 solar mass) companion and also inflating a local nebula\(^ \dagger \) confined by the ram pressure of the interstellar medium. We have detected X-ray emission from this system, using the Rosat satellite. X-ray emission is expected from the pulsar magnetosphere and the two shocks of the pulsar wind, one at the companion and the other inside the nebula. Our observations show that less than 20% of the pulsar’s spin-down luminosity can be carried away by electrons and positrons with Lorentz factor \(\gamma \approx 10^7\), and less than 5% by electrons and positrons with \(\gamma \approx 10^3\). Neither of these fluxes can provide the penetrating flux required to heat the companion’s photosphere. These observations and those in the accompanying paper by Fruchter et al.\(^ \dagger \) represent the first direct diagnostics of the relativistic wind from a weakly magnetized pulsar, and suggest that the wind differs substantially from that of the more highly magnetized Crab pulsar.

The composition of the relativistic wind, which removes energy at a rate \(\epsilon_{\text{wind}} = 1 \Omega\) as the pulsar spins down, is poorly known. Many models\(^ \dagger \) of pulsar magnetospheres suggest that this wind is primarily electromagnetic radiation, with little relativistic particle content. In contrast, the Crab pulsar reveals nebular radiation that is most easily understood\(^ \dagger \) as being produced when a wind dominated by ultrarelativistic electrons and positrons (hereafter ‘electrons’ should be taken to mean \(e^+\) and \(e^-\)) passes through a reverse shock. It has been argued\(^ \dagger \) that these points are reconciled if the pulsar wind becomes increasingly particle-dominated as it travels outwards, with magnetic reconnection increasing the mean particle Lorentz factors \(\gamma\).

The eclipsing pulsar system PSR1957+20 (refs 1, 9), consisting of a 1.61-ms pulsar and a 0.02 solar mass (\(M_\odot\)) dwarf companion separated by \(a = 1.7 \times 10^{13}\) cm, and surrounded by a \(\approx 10^6\) cm bow shock nebula\(^ \dagger \), offers new opportunities to probe the composition of a pulsar wind. Energetic electrons from the pulsar may produce X-rays, either in the pulsar magnetosphere or through interactions with the atmosphere of the companion or with the interstellar medium (ISM). We were interested in whether the wind from a weakly magnetized pulsar such as PSR1957+20 (surface magnetic field \(B_{\text{sur}} \approx 2 \times 10^9\) G) differs from the wind from a highly magnetized pulsar such as the Crab (\(B_{\text{sur}} \approx 4 \times 10^{12}\) G). We therefore observed PSR1957+20 with the Position Sensitive Proportional Counter\(^ \dagger \) (PSPC) aboard the X-ray satellite Rosat.

X-ray emission is seen close to the pulsar timing position\(^ \dagger \) (Fig. 1) with \(\Delta \alpha \approx -0.42\) s, \(\Delta \delta = 7\) arcsec (differences are in the sense of pulsar timing position minus that given by PSPC). These differences are within the expected (systematic) Rosat position error of 15 arcsec (90% probability error radius). A point X-ray source located 452 arcsec to the north of the pulsar, at \(\alpha(2000) = 19\,59\,53.33\) and \(\delta(2000) = 20\,40\,42.62\) (outside the field of view of Fig. 1) appears to coincide with a visible 11.3 mag star in the Hubble Space Telescope (HST) guide-star catalogue\(^ \dagger \). Many stars have associated X-ray emission, mainly due to chromospheric emission. Assuming that the association is real allows us to improve the absolute astrometry of the PSPC image. The new location of the X-ray peak results in \(\Delta \alpha = -0.22\) s, \(\Delta \delta = -6\) arcsec. Within the systematic error of the PSPC (2 arcsec) and the uncertainty due to Poisson statistics (3.5 arcsec), the X-ray peak is consistent with the pulsar timing position.

To first order, the X-ray emission contours are consistent with those of a point source. (The emission 72 arcsec north of the pulsar can be attributed to a star of visual magnitude \(V = 10.9\),

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