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Functional mammary gland development and oncogene-induced tumor formation are not affected by the absence of the retinoblastoma gene

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Loss of cell cycle regulation in mammary epithelium results in impaired mammary gland development and neoplasia. We investigated the consequences of the absence of pRb in mammary epithelial cells during normal development and in mice that express an oncogene in the mammary epithelium. Since pRb-deficiency results in embryonic lethality, we transplanted pRb-null mammary anlagen into wild hosts. pRb-deficient mammary epithelia were capable of functional differentiation in term animals and they regenerated a differentiated gland even after multiple pregnancies. In serial transplantations no significant differences were found in outgrowth of pRb-deficient and wild type epithelia indicating that the absence of pRb does not lead to transformation. Likewise the effect of a TGFβ1 transgene was not altered in the absence of pRb. The susceptibility of mammary epithelium to form tumors was assessed in three different models. No differences in tumor incidence were found between wild type and Rb +/− WAP-int3, MMTV-PyMT transgenic and *Brcal*−/− epithelia. These results demonstrate that the absence of pRb does not affect normal mammary gland development and tumorigenesis in three different mouse models investigated and suggest that loss of more than one member of the pRb pathway is required to induce mammary tumors. *Oncogene* (2001) **20,** 7115–7119.

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The retinoblastoma gene product pRb is an essential regulator of the cell cycle (Weinberg, 1995). However, it has not been possible to study its role in mammary epithelial cells because pRb-null mice die during embryonic development shortly after the mammary anlage is formed. We have now investigated the role of pRb through transplantations of pRb-deficient embryonic mammary anlagen into cleared fat pads of wild type hosts. This permitted studies on the function of pRb in postnatal mammary development and tumorigenesis. We also evaluated the role of pRb in the response to transgenic TGFβ expression, a process in which cell cycle regulation by pRb is thought to be important. Since mutations of *RB* are frequently found in human tumors of different origins (Dublin *et al*., 1998; Gorgoulis *et al*., 1998; Marsh and Varley, 1998) we also investigated whether the loss of pRb predisposes mammary epithelial cells that express an oncogene to malignancy.

To avoid graft rejection, the *Rb-1*$^{\Delta 20}$ mutation (Lee *et al*., 1992) was introduced into the FVB/N mouse strain by backcrossing for more than six generations. In this genetic background embryonic lethality of pRb-deficient mice occurred at around day E13. Embryos from E12.5 litters derived from hemizygote matings were used to dissect mammary anlagen, which were transplanted into cleared fat pads of FVB/N virgin female mice. When mammary anlagen from wild type (+/+), hemizygous (+/−) and homozygous *Rb-1*$^{\Delta 20}$(−/−) embryos were transplanted 55 to 68% gave rise to epithelial outgrowths in the fat pad (Table 1). Nine to 16% of these transplants developed into a cyst that contained only epidermal structures because technically it was not possible to separate the epidermis from the embryonic mammary anlagen and the cultures contained both types of tissues. The remaining 45 to 60% of transplanted glands contained functionally differentiated mammary epithelial tissue (Table 1). In whole mount preparations no differences were detected in the density and amount of alveoli between the different genotypes. Mammary tissue derived from the transplanted pRb-null epithelium displayed morphological and biochemical criteria of functional differentiation. The cells contained secretory vesicles and fat droplets, and the lumina were expanded and filled with secreted material (Figure 1A). Both β-casein and WAP gene expression was normal (Figure 1B). These results show that pRb is not required for growth and functional differentiation of mammary epithelial cells after repeated cycles of involution. Transplants harvested after nine consecutive pregnancies were indistinguishable from first term transplants (Figure 1).

Serial transplants are the most direct test to measure the proliferative potential of mammary tissue and

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Table 1 Development of embryonic transplants of wild type and pRb-mutant mammary glands

Genotype of transplant	+/+	+/−	−/−
# of transplants	31	67	38
# of mammary epithelial outgrowths	16 (52%)	40 (60%)	17 (45%)
# of epidermal cysts	5 (16%)	6 (9%)	4 (10%)
# empty fat pads	10 (32%)	21 (31%)	17 (45%)

Two to 3 embryonic glands were co-cultured on a piece of ventral mesenchyme from the same embryo for 16 h as described (Robinson *et al*., 2000). The cultures were transplanted into the inguinal (fourth) mammary fat pad of FVB/N hosts whose endogenous mammary epithelium had been removed surgically (DeOme *et al*., 1959). In order to ensure that the outgrowth was created from the embryonic epithelial transplant, complete removal of the endogenous epithelium was carefully monitored by reserving the excised part of the host gland for whole mount staining. Only cleared fat pads in which the entire ductal tree of the host was contained in the excised part of the fat pad were used for analysis. The embryonic origin of the transplanted epithelium was further ascertained by PCR analysis of DNA extracted from a small piece of the outgrowth at the time of harvest. At low cycle numbers a stronger band was amplified from the transplanted pRb-deficient epithelium compared to the wild type *Rb* band derived from the stroma (data not shown). Hosts were mated 8 weeks after transplantation. The transplants were harvested the morning after delivery of pups and prepared for whole mount evaluation of the outgrowth

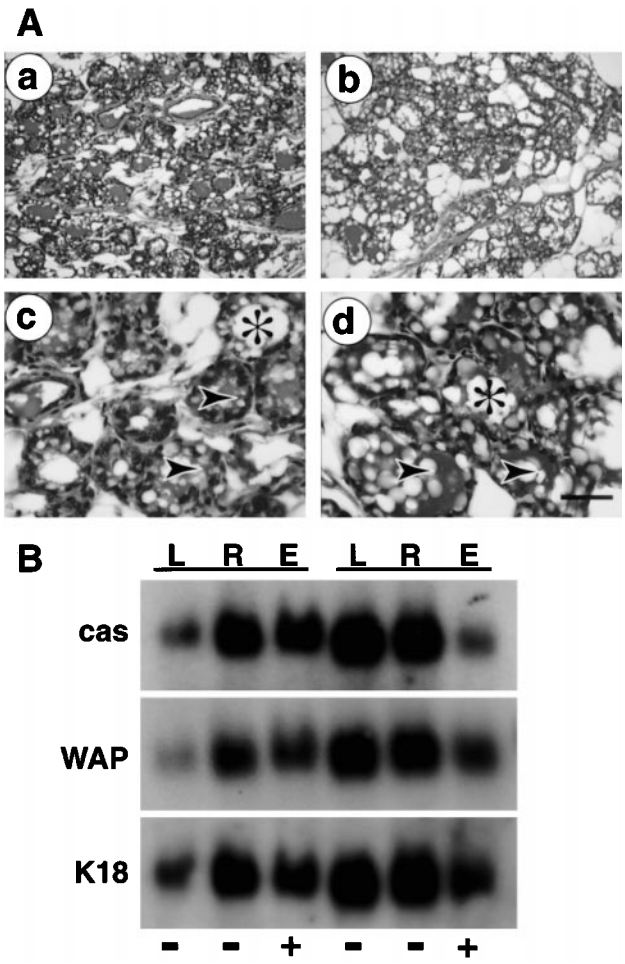

Figure 1 Normal histological development of transplanted pRb-deficient epithelium. (**A**) Mammary epithelia from pRb-deficient (a and c) and wild type embryos display functional differentiation after transplantation into wild type hosts. Milk is secreted to the lumen (*). The epithelial cells are polarized and contain fat globules (arrows). The transplant in a and c was harvested after delivery of nine litters. The transplant in b and d was harvested after the first litter. (**B**) Expression of milk proteins in transplanted pRb-deficient epithelia (R and L indicate two transplants in the left and right gland) and the endogenous (E) thoracic gland of the same animal. Twenty micrograms of total RNA prepared from tissue homogenates (Chomczynski and Sacchi, 1987) were separated in a formaldehyde gel and blotted onto nylone membrane. The probes for detection of milk proteins were used as described before (Robinson *et al*., 1998). Hybridization of cytokeratin 18 served as a loading control. Scale bar 100 mm in a and b; 30 μm in c and d

provide information on a transformed phenotype (Daniel *et al*., 1968). We carried two different lines of pRb-deficient embryonic transplants and one line of wild type epithelium through this regimen. Only small differences were observed between the two genotypes of the epithelia in the first round of re-transplants. Approximately 85% of the transplants were able to fill the fat pad to over 50% (Table 2). Pieces of tissue derived from a transplant that had given full outgrowth were transplanted again, and 36% of the wild type cells gave an outgrowth that expanded through more than 50% of the fat pad. The two pRb-deficient lines displayed divergent results. While in one line 62.5% of the transplants filled more than half the fat pad and 6% gave no outgrowth, on 17% of the transplants in the other line filled 50% of the fat pad and 83% gave small or no growth (Table 2). These results indicate that pRb-deficiency does not alleviate the gradual loss of regenerative capacity and pRb-deficient transplants are subject to senescence upon serial transplantation to a similar extent as the wild type cells.

TGFβ exerts a wide range of effects in different systems that foremost affect cell cycle regulation (Massague *et al*., 2000). We therefore decided to investigate the response of pRb-deficient mammary epithelia in a situation where they are exposed to a challenge by TGFβ. Ectopic expression of an activated form of TGFβ1 in mammary epithelia cells of transgenic mice inhibits development of a full ductal tree (Jhappan *et al*., 1993). This is due to a high rate of cell death during pregnancy and leads to the induction of early senescence of mammary epithelial stem cells (Kordon *et al*., 1995). Since pRb is a molecular target for TGFβ in cell cycle control, it can be hypothesized that TGFβ mediated inhibition of cell growth and senescence is abrogated in the absence of pRb. To investigate this possibility we crossed the WAP-TGFβ1 transgene into pRb-mutant animals and performed mammary epithelial transplants. No differences were seen in the morphology of TGFβ1 overexpressing mammary epithelia (Figure 2). In both cases ductal trees were sparse, and development of alveoli at term was inhibited and morphologically indistinguishable. The alveoli appeared small and functional differentiation was attenuated as demonstrated by the lack of fat

Table 2 Clonogenic capacity of serial transplants

Genotype	*2nd generation*		*3rd generation*	
Outgrowth	> = 50%	< = 20%	> = 50%	< = 20%
+/+	82% (9/11)	18% (2/11)	36% (5/14)	57% (8/14)
−/−	87.5% (7/8)	12.5% (1/8)	62.5% (10/16)	6% (1/16)
−/−	83% (10/12)	17% (2/12)	17% (1/6)	83% (5/6)

The percentages of transplants that gave rise to an outgrowth that filled over half of the fat pad (50% filling) and those that gave small (20% or less filling of the fat pad) outgrowths in the second and third transplant generation are given. In parentheses are the numbers of transplants that gave any kind of outgrowth and the total number of transplants that were performed. Small pieces of mammary tissue from embryonic (first generation) transplants that were fully developed after puberty and pregnancy were grafted into the epithelial-free fat pad of a host and permitted to repopulate the host fat pad. This procedure was repeated after the second-generation outgrowths had passed through 8 weeks of growth in the virgin host and pregnancy to establish third generation outgrowths

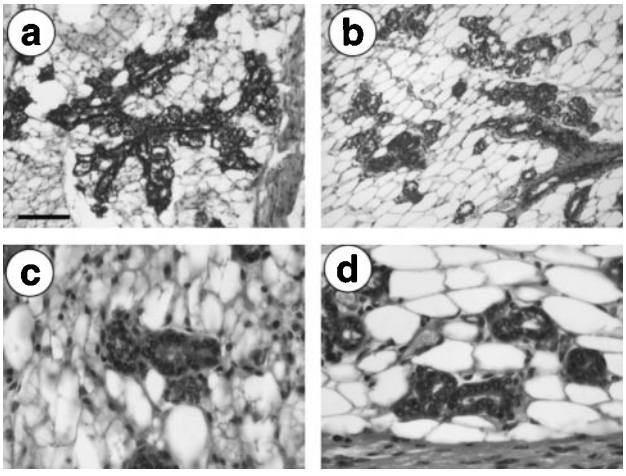

Figure 2 Attenuated development of transplanted WAP-TGFβ1 transgenic epithelium. The same inhibition of epithelial differentiation is observed in pRb-deficient (a and c) as in pRb heterozygous (b and d) WAP-TGFβ1 transgenic epithelial transplants. Only a few epithelial cells show secretory activity. The majority of the epithelial cells are unpolarized and contain large nuclei. The transplant in a and c was harvested after delivery of the first litter. The transplant in b and d was harvested after the fourth litter. Scale bar 100 μm in a and b; 30 μm in c and d

droplets and secreted material in the lumina. The secretory epithelial cells were round; they contained large nuclei and appeared not polarized (Figure 2). This phenotype was also observed in transplants that were harvested after nine pregnancies (data not shown). These results demonstrate that pRb is not required for TGFβ action in mammary epithelial cells. This differs from the observation in embryonic lung cultures where an interaction between TGFβ and pRb was found (Serra and Moses, 1995). pRb-deficient fibroblast were also shown to become insensitive to the growth inhibitory effect of TGFβ when grown at high density (Herrera *et al*., 1996). This indicates cell specificity in the regulation of cell cycle progression by pRb.

The effect of pRb on mammary tumor formation was tested by assessing transgenes that induce mammary tumors with high frequency. We crossed three mouse lines that are prone to mammary tumors into the pRb-deficient background and compared tumor incidence in wild type and mice hemizygous for *Rb*. We reasoned that the intact *Rb* allele might be lost in this situation and lead to faster development of tumors. Three different models for mammary tumors were investigated. (1) The WAP-int3 transgene, a truncated form of the *Notch4* gene, which was originally identified as an oncogene by activation through mouse mammary tumor virus (MMTV) insertions (Gallahan *et al*., 1996). (2) Mice that carry the MMTV-polyoma virus middle T (PyVMT) transgene (Guy *et al*., 1992). (3) The deletion of the tumor suppressor gene *Brca1* in mammary epithelial cells leads to tumors after a long latency period which is decreased in a *p53*-hemizygous background (Xu *et al*., 1999). The median period for development of tumors in mice carrying the WAP-int3 transgene with either two intact *Rb* alleles ($n = 11$) or one *Rb*-null allele ($n = 13$) was 167 and 168 days ($P = 0.684$), respectively (Figure 3a). In mice carrying the MMTV-PyVMT transgene and two intact *Rb* alleles half of the mice developed tumors by 102 days ($n = 9$), while the latency

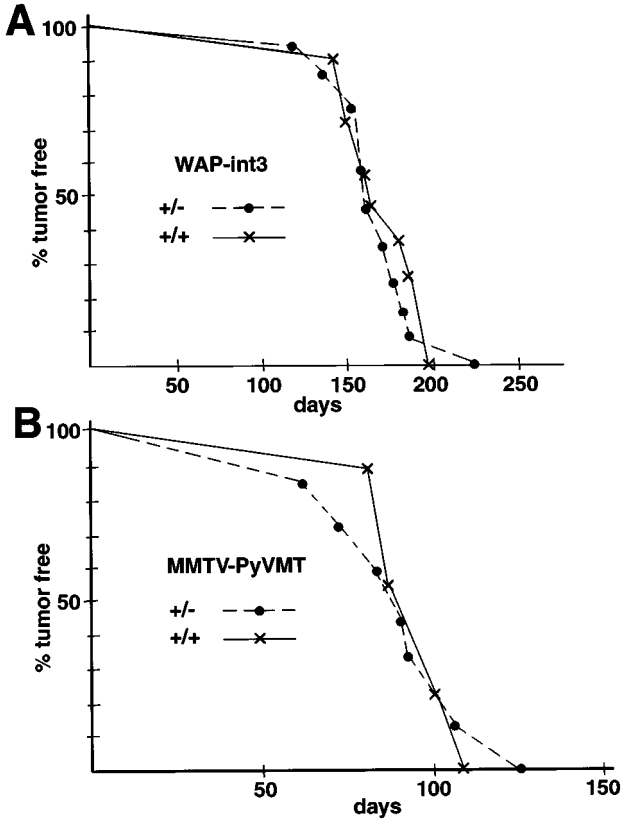

Figure 3 Tumor development in wild type and pRb+/− mice. Mice with one or two intact alleles of *Rb* and either int3 (**A**) or PyVMT (**B**) transgene were monitored weekly and sacrificed when a tumor of 1 cm diameter was detected. The data were plotted as percentage of tumor free animals against time in days

period was 93 days in the presence of one *Rb*-null allele ($n=7$)($P=0.768$) (Figure 3b). These differences are not significant. Nevertheless, these timeframes might be too short to allow for loss of the second *Rb* allele. In order to directly assess tumor development also in *Rb*-null epithelium, we transplanted WAP-int3 embryonic anlagen with the different *Rb* genotypes. Preliminary data show that irrespective of the *Rb* genotype, the tumor latency was extended. The tumor latency, which was approximately 170 days in WAP-int3 transgenic animals was prolonged when embryonic anlagen were transplanted (290 days for *Rb*+/+ WAP-int3 ($n=5$), 294 days for *Rb*+/− WAP-int3 ($n=5$) and 260 days for the *Rb*−/− WAP-int3 ($n=2$) transplants). The reason for the delayed onset of tumors in the embryonic transplants is not understood. Nevertheless, no acceleration of tumor formation was observed in the absence of one or both *Rb* alleles. A cohort of mice carrying one deleted and one conditionally targeted *Brcal* gene (Xu *et al*., 1999), in which this exon is excised in mammary epithelial cells by Cre recombinase under regulation of the WAP gene promoter (Wagner *et al*., 1997), were monitored for mammary tumor development. The mice were bred and delivered three to four litters to ensure expression of the recombinase and efficient inactivation of *Brca1*. All six mice that were hemizygous for *Rb* developed a wasting syndrome at an average age of 13.7 months. Upon autopsy these mice were found to have developed pituitary tumors as was described before (Hu *et al*., 1994). No overt signs of hyperplasia or nodules were detected in the mammary glands. In three of the mice the mammary ducts were regressed as would be expected in mice that were no longer fertile and older than 1 year. Three of the mice showed ducts that were decorated with alveoli, indicative of elevated prolactin levels secreted by the pituitary tumor. After the much longer latency times in this experiment loss of *Rb* ensued in the pituitary but no co-operation between loss of *Brca1* and *Rb* haploinsufficiency was observed in mammary epithelium. No mammary tumors were detected in nine WAP-Cre transgenic mice with one deleted and one conditional *Brca1* gene and two intact *Rb* alleles after 16 months. Taken together these data indicate that in three different model systems, which generate mammary tumors, there was no acceleration of tumor formation when only one intact allele of *Rb* was present. A connection between pRb and Brca1 in cell cycle control has been suggested in tissue culture cells. Overexpression of Brca1-induced growth inhibition is abrogated if these cells also overexpress the HPV E7 protein which inhibits the activity of pocket proteins (Aprelikova *et al*., 1999). Our data show that none of the oncogenic events are accelerated in *Rb*-hemizygous mammary cells and indicate that pRb does not interact with the tested oncogenic pathways to induce a loss of cell cycle control and transformation.

A similar transplantation approach has been used to investigate the development and tumorigenesis of pRb-deficient prostate epithelium (Wang *et al*., 2000). Embryonic pelvic organs were transplanted under the kidney capsule of adult male hosts. The prostatic tissue developed in these transplants was further analysed as transplants. In untreated hosts the transplants developed mild hyperplasia while development of hyperplasia and carcinoma were found in hosts that were treated with testosterone. In this case, the absence of pRb predisposed prostatic epithelium to increased cell proliferation and hyperplasia.

Four other methods have been used to evaluate development of pRb-deficient tissues. (1) Primary cultures of cells and tissues derived from gene knockout embryos; (2) production of chimeric mice containing pRb-deficient embryonic stem cells that contribute in a random fashion to different tissues; (3) inactivation of the pRb protein by viral oncoproteins such as human papilloma virus E7, adenovirus E1A or the simian virus T antigen (SV40Tag); (4) tissue specific inactivation of the gene (Vooijs *et al*., 1998). The chimera experiments demonstrated that pRb-deficient cells participated in the formation of mammary glands, and no obvious defects in overall glandular development were observed (Maandag *et al*., 1994). Our finding that pRb-null mammary anlagen can develop into differentiated mammary epithelium upon grafting into wild type hosts supports these findings. Further, the transplants underwent normal apoptosis and involution-mediated remodeling and we did not observe any alterations in their histological appearance or signs of neoplasia even after nine consecutive pregnancies. These results were unexpected since pRb is a crucial component of cell cycle control and its deregulation or inactivation is associated with many tumors. After each cycle of pregnancy, which is characterized by high levels of cell proliferation, a massive wave of apoptosis occurs in the mammary gland. An imbalance of these processes results in mammary tumors as demonstrated in several transgenic lines of mice in which the SV40-Tag is expressed in mammary epithelial cells (Tzeng *et al*., 1993; Maroulakou *et al*., 1994; Li *et al*., 1996). The inactivation of pRb by homologous recombination and through transgenic expression of SV40-Tag differ in two aspects. While pRb is never present in transplanted pRb-deficient mammary epithelial cells, inactivation by transgenic SV40-Tag occurs preferentially in the epithelium during pregnancy. In addition, SV40-Tag ablates the activities of all the pocket proteins and also inactivates p53.

Recently it was shown that inactivation of all three members of the pocket protein family in embryonic stem cells and mouse embryonic fibroblasts has a more profound effect on cell cycle control than single or double knock out of the family members (Dannenberg *et al*., 2000; Sage *et al*., 2000). Triple knock out cells were resistant to G1 arrest induced by DNA damage, contact inhibition and serum starvation and became immortal in culture. The ability to differentiate was severely impaired in triple knock out embryonic stem cells (Dannenberg *et al*., 2000; Sage *et al*., 2000). This was also demonstrated in the adipogenesis of 3T3 cells lacking one or more pocket protein family members.

While pRb-deficient 3T3 cells differentiated poorly no such defects were found in cells that lacked p107 and p130 (Classon *et al*., 2000). Taken together these observations clearly demonstrate some redundancy and cell specificity in the function of these proteins to regulate cell cycle progression and differentiation. Our results demonstrate that the absence of pRb does not affect mammary epithelial cell proliferation, differentiation and oncogene-induced tumorigenesis. This further supports the notion that mutation of two or more members of the pRb pathway such as cyclins, cyclin-dependent kinases or their inhibitors is required for cell transformation in mammary epithelial cells.

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