RB1 in Cancer: Different Mechanisms of RB1 Inactivation and Alterations of pRb Pathway in Tumorigenesis

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Loss of RB1 gene is considered either a causal or an accelerating event in retinoblastoma. A variety of mechanisms inactivates RB1 gene, including intragenic mutations, loss of expression by methylation and chromosomal deletions, with effects which are species–and cell type-specific. RB1 deletion can even lead to aneuploidy thus greatly increasing cancer risk. The RB1gene is part of a larger gene family that includes RBL1 and RBL2, each of the three encoding structurally related proteins indicated as pRb, p107, and p130, respectively. The great interest in these genes and proteins springs from their ability to slow down neoplastic growth. pRb can associate with various proteins by which it can regulate a great number of cellular activities. In particular, its association with the E2F transcription factor family allows the control of the main pRb functions, while the loss of these interactions greatly enhances cancer development. As RB1 gene, also pRb can be functionally inactivated through disparate mechanisms which are often tissue specific and dependent on the scenario of the involved tumor suppressors and oncogenes. The critical role of the context is complicated by the different functions played by the RB proteins and the E2F family members. In this review, we want to emphasize the importance of the mechanisms of RB1/pRb inactivation in inducing cancer cell development. The review is divided in three chapters describing in succession the mechanisms of RB1 inactivation in cancer cells, the alterations of pRb pathway in tumorigenesis and the RB protein and E2F family in cancer.
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The retinoblastoma (RB) gene RB1, located at chromosome 13q14.2, is part of a larger gene family that includes two other RB-related genes designated as retinoblastoma-like 1 (RBL1) and retinoblastoma-like 2 (RBL2). RBL1 is located at chromosome 20q11.2, a region of special interest because of its association with some myeloid disorders (Claudio et al., 2002); RBL2 is located at chromosome 16q12.2, an area in which deletions or loss of heterozygosity have been found in several human neoplasms and correlated with clinical aggressiveness (D'Andrilli et al., 2004). These three genes are structurally related, each has the ability to suppress tumor cell growth in vitro and several results suggest that, to manifest the fully transformed phenotype of mammalian cells, their simultaneous inactivation may be required (Modi et al., 2000). RB1, RBL1, and RBL2 genes encode structurally related proteins belonging to the RB protein family, which are indicated as pRb, p107, and p130. These proteins share extensive structural homology and contain a conserved domain indicated as "pocket" (Chan et al., 2001), which was originally identified as a region required to bind viral oncoproteins, as adenovirus E1A (Ad-E1A), Simian virus 40 large T antigen (SV40 LT-antigen) and human papilloma virus E7 (HPV-E7; Moran, 1993). The pocket domain has been thereafter described as required for the physical interaction of RB proteins with a variety of cellular proteins believed to work with RB proteins in transcriptional regulation, including human D-type cyclins (Dowdy et al., 1993), BRG1 (Dunaief et al., 1994), and HDAC1 (Brehm et al., 1998).

More than 750 reviews have been published on RB proteins and more than 150 focused on RB proteins and cell cycle control. Among them we want, in particular, to highlight the comprehensive reviews of Cobrinik (2005) and Henley and Dick (2012). These reviews extensively describe the key roles played by the RB proteins in regulating the advancement of the cell cycle from G1 to S phase through negative regulation of E2F transcription factors (E2Fs) and cyclin dependent kinases (CDKs). The reviews report a great number of paper describing the hypophosphorylated state of RB proteins (with binding and inhibition of the E2F transactivation domain) and their hyperphosphorylated state (with the release of E2F and the expression of genes that mediate S phase entry). They also describe how the E2Fs deregulation not only increases cell proliferation, but also induces apoptosis, senescence, checkpoint defects and altered DNA damage response, even

preventing differentiation. The reviews also analyze the regulation determined by pRb on cell cycle transitions through E2F-independent mechanisms and its effect on the spatial organization of genomic replication. The roles of RB proteins in organisms as distantly related as humans, plants and insects are reported.

As RB proteins are central to the regulation of cell proliferation, deregulation of cell cycle control requires the inactivation of their growth regulatory functions to favor cancer onset.

Here, by our review, we wanted emphasize the importance of the mechanisms of RB1/pRb inactivation for inducing cancer cell development.

Brief history of the retinoblastoma disease and gene

The identification of the retinoblastoma susceptibility gene (RB1)—the first tumor suppressor gene to be identified—was a milestone in understanding cell cycle control and cancer genetics (Wang et al., 1994; Sellers and Kaelin, 1997). More than 50 years ago, a children's tumor (the retinoblastoma) was realized to sporadically occur in some patients, but to be inherited in others (Falls and Neel, 1951). After that, one important piece of the tumor suppressor gene story began with the studies of Alfred Knudson at the MD Anderson Cancer Center and Tumor Institute in Houston, on hereditary patterns for childhood retinoblastoma. In this center, in 1971, Knudson, after years of observing cases of childhood cancer and the likelihood that certain heredity patterns played a role in them, focused on retinoblastoma, a type of pediatric eye cancer. The studies—developed examining the family histories of 48 patients with this rare cancer of the retinas—created the foundation for the tumor suppressor hypothesis. Since then, retinoblastoma has represented a prototype biological model for the study of a class of oncogenes, in which tumor-predisposing mutations are recessive to wild-type alleles (Vogel, 1979). Now, Knudson is internationally recognized for his "two-hit" model of cancer causation (Knudson, 1971), which explained the relationship between the hereditary and non-hereditary forms of a cancer and predicted the existence of tumor-suppressor genes that can suppress cancer cell growth. According to this model, two mutational events or two hits are required for tumor onset: an individual may have inherited a germline mutation from a parent and that would constitute the first "hit" leading to the cancer, then the disease would develop only after a second mutational event, or second "hit," produced either spontaneously or otherwise. Thus, to transmit retinoblastoma predisposition to offspring, RB1 mutation must be present in germ-line cells and one allele mutation must be transmitted as an autosomal dominant trait (Bamne et al., 2005). Tumor development will be initiated by inactivation of the second RB1 allele (Valverde et al., 2005; Macpherson, 2008); indeed biallelic mutations in RB1 have been recognized as the causative genetic alteration for retinoblastoma and, accordingly, introduction of RB1 into RB1−/− cells reduces their ability to promote malignant transformation (Richter et al., 2003). However, since mutations in the second allele can occur independently in several cells, multiple tumor foci arise in most individuals who have inherited a predisposing RB1 mutation and this predisposes patients to a variety of other malignancies (Monteiro, 2003). In short, the manifestation and transmissibility of retinoblastoma depend on the nature of the first mutation, its time in development, and the number and types of cells that are affected.

It is very interesting to note that retinoblastoma, a relatively rare cancer [approximately 4% of childhood cancer and less than 1% of all human cancers (Abramson, 2005)], has contributed, more than others, to the understanding of cancer, dramatically changing the way cancer is studied and understood.

Studies on retinoblastoma also produced important scientific advancement in the field, as the identification of the RB1 gene locus in chromosome region 13q14.2 (Lalande et al., 1984), the cloning of a DNA sequence with many of the properties predicted for the retinoblastoma susceptibility locus (Friend et al., 1986), and the identification and subsequently cloning of a 4.7 kb RB1 transcript (Lee et al., 1987).

However, the large size of RB1 gene (about 200 kb) and its multiple dispersed exons—27 exons with two of the introns being extremely large (35 and 70 kb)—(Hong et al., 1989), strongly complicated molecular screening strategies. Indeed, to permit the molecular detection of chromosomal translocations associated with retinoblastoma, it was necessary to construct a long-range restriction map, with the use of infrequently cutting restriction enzymes, field inversion gel electrophoresis and two cloned fragments from the ends of RB1 gene (Higgins et al., 1989).

Although the two-hit hypothesis suggested that the first few years following birth reflects the time-interval for homozygous RB1 mutation, a large number of cytogenetic and comparative genomic hybridization studies have shown that RB1 inactivation is not sufficient for determining retinoblastoma. These studies have suggested that mutations of both alleles of the RB1 gene are necessary for retinoblastoma tumor initiation (Wang et al., 1994) but not sufficient for malignant transformation (Sellers and Kaelin, 1997). Thus, additional mutational events (three hits) are required for RB1−/− cells to progress into a fully malignant tumor (Corson and Gallie, 2007). In particular, in mouse models of retinoblastoma, retinal tumors only develop when RB1 is lost together with at least another RB-related gene/protein (Robanus-Maandag et al., 1998); in humans, loss of both copies of RB1 does not lead directly to retinoblastoma, but to retinoma (Dimaras et al., 2008) with a low level genomic instability and high expression of the senescence-associated proteins as the inhibitor of cyclin-dependent kinase 4a (p16INK4a) and the RB family member p130. This suggests that other family members can enforce cell cycle exit and inhibit tumorigenesis in the absence of RB1. However, as progressive genomic instability leads to highly proliferative, clonal, and aneuploid retinoblastomas, it is difficult to clinically observe stable retinoma (Dimaras et al., 2008).

Children with retinoblastoma have a high risk of developing second cancers as soft tissue sarcoma, osteosarcomas, melanoma, Hodgkin disease, leiomyosarcoma and prostate, breast, brain, lung and buccal cavity (salivary gland and tongue) cancers (Abramson, 2005). However, the tumor more frequently associated to retinoblastoma is osteosarcoma with individuals affected by hereditary retinoblastoma having an approximately 1,000 times higher incidence of this tumor (Berman et al., 2008), with loss of heterozygosity of RB1 locus (60–70%) representing a poor prognostic factor (Feugeas et al., 1996; Alonso et al., 2001).

Mechanisms of RB1 Inactivation in Cancer Cells

Cancer is a heterogeneous disease whose initiation and progression is promoted by the aberration of genes that regulate the most important cellular processes (proliferation, adhesion, differentiation, death) devoted to maintain the integrity of complex organisms, and RB1 loss is an important step in cancer development (Friend et al., 1986).

There are a large variety of genetic, epigenetic and chromosomal changes that accumulate in cancer cells, with the cells of most malignancies even showing aneuploidy (D'Urso et al., 2010). However, although it is well known that the majority of human cancers progress through the gradual accumulation of genetic and epigenetic alterations, the genetic mechanisms that initiate carcinogenesis are not well understood.

Chromosomal abnormalities, RB1 deletion, and cooperating mutational events

Constitutional chromosome alterations, deletion of tumor suppressor genes and overexpression of oncogenes have been increasingly regarded as key initiating events in human cancers (Hansen and Cavenee, 1987; Chin et al., 2011). A study of cancer incidence in a follow-up of 2561 patients with constitutional autosomal chromosome deletions performed during the period 1965–2002 (Swerdlow et al., 2008), describes 13q chromosomal-deletions in retinoblastoma cells, in sporadic as well as constitutional cases (the latter having the deletion in all cells of the body). This study strongly suggests that, when constitutionally present, this deletion is the reason for greatly increased risk of cancer. Moreover, genetic linkage studies of chromosomal abnormalities in hereditary retinoblastoma (focused on chromosome 13q14) supported the idea that retinoblastoma is a recessive cancer where the abnormal chromosome is inherited and the corresponding wild type chromosomal segment is lost in tumor cells (Godbout et al., 1983). Today, it is known that RB1 loss can lead to chromosome segregation defects through a number of disregulations, among which misregulation of the expression of genes important for centrosome duplication, for mitotic checkpoint control or for eterochromatin structure formation and maintenance (Sage and Straight, 2010).

As centrosome maintains genomic integrity by enforcing euploidy (Adon et al., 2010), centrosome amplification determines aberrant and multipolar mitotic spindles, by increasing frequency of chromosome segregation errors, aneuploidy, and chromosome instability. This contributes to cancer biogenesis and progression by triggering reduced expression of tumor suppressors and overexpression of oncogenes. One of the mechanisms contributing to centrosome amplification is deregulated centrosome duplication triggered by the G1-CDKs (Adon et al., 2010); centrosome amplification, is also associated with mutation or loss of function of genes as TP53, STK15, RB1, and BRCA1 (Albertson et al., 2003). Moreover, several studies demonstrated that the RB1 gene isolated from retinoblastoma tumors contains intragenic mutations with premature stop codons, in-frame deletions and point substitutions (DeCaprio, 2009).

Mutations in oncogenes and tumor suppressor genes could play cooperative roles in initiation and progression of cancer (Fig. 1). Large cancer-associated chromosomal deletions could arise from selective pressure to attenuate the activity of multiple genes (Xue et al., 2012). For example, since RB1 loss leads to enhanced cell death, whereas TP53 loss facilitates cell survival, the simultaneous disruption of RB1 and TP53 genes seen in many tumors, suggest a cooperation between these
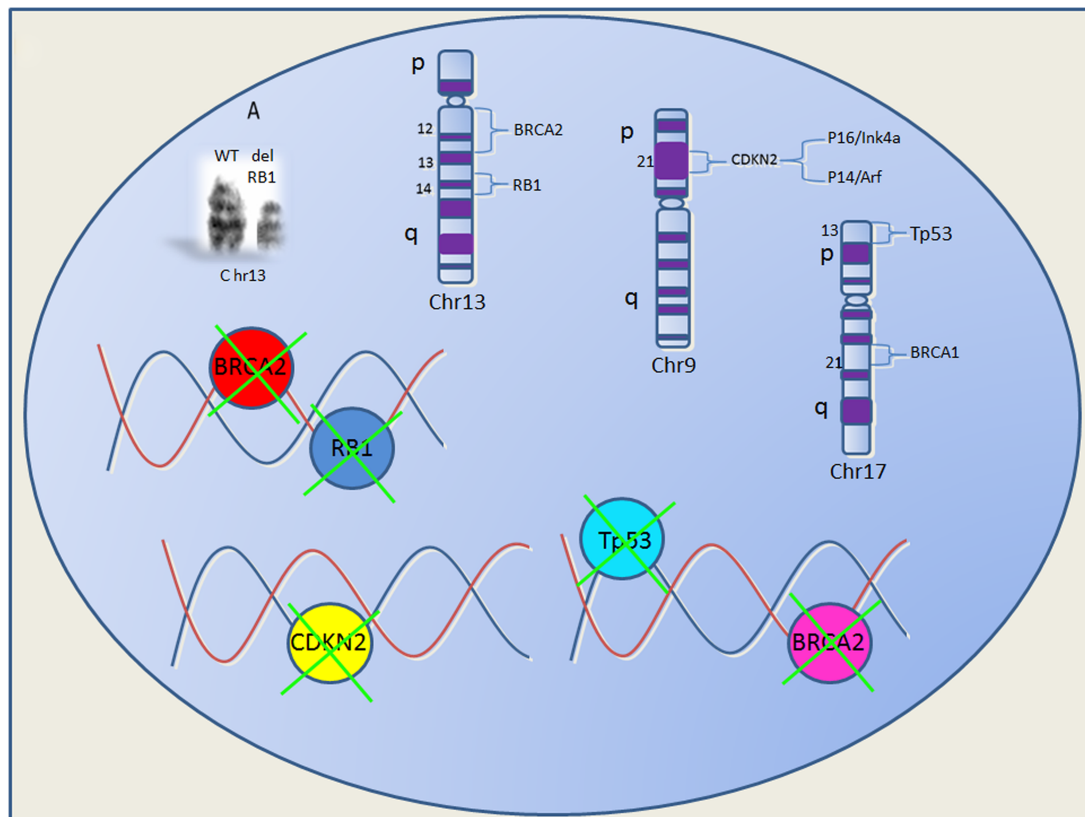

Fig. 1. Genetic perturbations in RB1, CDKN2, Tp53, and BRCA1 and BRCA2 locus cooperate in promoting tumor progression. Chromosomal abnormalities affecting RB1 in combination with CDKN2, Tp53, and BRCA1 and BRCA2 delections cooperate in the establishment of a complex scenario in which loss of function of key genes contributes to cancer initiation and progression. Using human retinoblastoma samples and mouse models, it has been reported that RB1 gene deletion (described in A) initiates a process of retinoblastoma development throughout cooperating mutational events involving chromosome 13, chromosome 9, and chromosome 17. This results in the deletion of the genes reported on the double DNA helix with consequent cancer progression.

losses in determining cancer (Plisiecka-Hałasa et al., 2008). Similar cooperations in determining cancer are suggested for the disruption of p53 and pRb pathways observed in most human cancer. In this regard, it is known that the CDKN2A/B locus is involved in both the pRb and p53 pathways by encoding both p16INK4a, a regulator of CDK4/6-mediated pRB phosphorylation, and p14ARF, a modulator of Mdm2-mediated degradation of p53 (Sharpless and DePinho, 1999). Moreover, it has been demonstrated that CDKN2A deletion plays an important role in the malignant behavior of a number of cancer as gastrointestinal tumors (Haller et al., 2008), malignant gliomas (Liu et al., 2011), mesothelioma (Jean et al., 2012) and others. Using human retinoblastoma samples and mouse models, it has been suggested that ARF is a key collaborator with pRb in retinoblastoma suppression since RB1 gene deletion initiates a process of cooperating mutational events between pRb and ARF in promoting mouse retinoblastoma (Conkrite et al., 2012). Comparing gene expression signatures of normal retinas and retinoblastoma tumors from a number of patients, microarray analysis associated with statistical and bioinformatic analyses, suggested that the genes differentially expressed in retinoblastoma mainly belong to DNA damage-response pathways, including breast cancer associated genes (BRCA1, BRCA2), ataxia telangiectasia mutated gene (ATM), ataxia telangiectasia and Rad3 related gene (ATR), E2F and checkpoint kinase 1 (CHK1) genes. In addition, novel pathways, such as aryl hydrocarbon receptor (AHR) signaling, polo-like kinases (Plks) and purine metabolism pathways are involved. Of particular interest appears the involvement of AHR, CHK1, and Plks, as several drugs that target these molecules are currently available (Ganguly and Shields, 2010). In addition, using genetically engineered mice, it has been shown (Szabova et al., 2012), that inactivation of RB1 induces surface epithelial proliferation with progression to stage I carcinoma, and that additional biallelic inactivation and/or missense TP53 mutation in the presence or absence of BRCA1/2 cause progression to stage IV disease. Moreover, as in human serous epithelial ovarian cancer, mice developed peritoneal carcinomatosis, ascites, and distant metastases. This strongly suggested a cooperation among RB1, TP53 and BRCA1 or BRCA2, confirming the scenario complexity of the molecular players involved in cancer initiation and progression.

Overall, the results suggest that in the absence of normal RB1 gene, genomic instability and chromosomal aberrations accumulate leading to tumor initiation, progression and metastasis. Thus, the identification of the combinations of mutations that collaborate in the development and progression of specific types of cancer can bring innovative contributions to cancer research.

Alterations of pRb Pathway in Tumorigenesis

The human retinoblastoma protein pRb is a 928 amino acids chain whose conserved pocket domain is a region that binds various critical protein interactors, many containing an LXCXE motif (Chan et al., 2001). This pocket domain has a small pocket region, consisting of A and B domains separated by a spacer region (Classon and Dyson, 2001), which acts as transcriptional repressor (Chow et al., 1996) and interacts with viral oncoproteins (Hu et al., 1990). The small pocket domain together with the C-terminal domain form the large pocket region which is the growth suppressing domain of the RB family proteins (Bremner et al., 1995). This pocket fragment permits the interaction of pRb with E2F family transcription factors allowing the suppression of their transcriptional activity and the control of cell proliferation (Hiebert et al., 1992).

Intrinsic checkpoints represent one of the major design of the cells to combat aberrant proliferation and to preserve genomic stability, and checkpoints elimination results in cell death, infidelity of chromosome transmission, or increased susceptibility to environmental perturbations, which can be the cause of human cancers (Lavia et al., 2003). Key oncogenic events in cancer can either directly perturb proteins that regulate progression through cell cycle, or indirectly alter cell cycle progression, through effects on pathways that impinge on the cell cycle, with the G1-S checkpoint being a cardinal process. As a consequence, cancer cells multiply when and where they should not and this permits maintenance of DNA damage and chromosomal imbalances with altered division time.

Because of its pocket domain, pRb is a multifunctional protein that can interact with a variety of proteins and this implicates that it can regulate not only the cell cycle but also other cellular activities among which DNA replication, cellular senescence, differentiation, and apoptosis (Knudsen and Knudsen, 2006). pRb participates in a regulatory network that governs the cellular response to antimitogenic signals and acts as the gatekeeper of the G1/S transition, with its deregulation constituting one of the hallmarks of cancer. Alterations of the pRb signaling pathway by activation of positive components as G1 cyclins and CDKs, inactivation of negative components as CDK inhibitors and p53, or by mutations in RB1 itself (Fig. 2), have been detected in virtually all human cancers.

pRb phosphorylation/inactivation is mediated by CDK4/6 whose overexpression represents one way to induce cancer. A number of results suggests the involvement of CDK4 gene in tumorigenesis, and among these are (i), the suppression of CDK4 can lead to terminal differentiation of erythroleukemia cells, whereas its overexpression can induce uncontrolled cell growth and malignant transformation (Xiong et al., 1993); (ii), the amplification and consequent overexpression of the CDK4 gene have been found in various cancers including different types of sarcomas and glioblastomas (Khatib et al., 1993; Collins, 1995); (iii), in human melanomas it has been identified a somatic point mutation of CDK4 (Wölfel et al., 1995) which prevented the binding of the CDK4 inhibitor p16INK4a, but not of p21 or of p27KIP1. This mutation can disrupt the cell-cycle regulation exerted by the tumor suppressor p16INK4a; and (iv), since CDK4 is inhibited by a series of inhibitory proteins (among which p16INK4a), the lack of INK4a function, also participates in this scenario (Perrone et al., 2005).

Thus, pRb can be functionally inactivated through disparate mechanisms and the constitutive pRb hyperphosphorylation is one of the major mechanisms (Chatterjee et al., 2004). This has also been shown in human osteosarcoma MG-63 cells, where aberrant gene expression keeps pRb protein constitutively inactivated by hyperphosphorylation which strongly contributes to uncontrolled cell proliferation (De Blasio et al., 2005); this also occurs in human hepatocellular carcinoma where pRb inactivation is associated with promoter methylation of the p16INK4a gene (Maeta et al., 2005), or in serous ovarian carcinomas where p53 and p16INK4a overexpression and low expression of p21WAF1/CIP1 permit to define the ovarian tumor grade (D'Andrilli et al., 2008).

The effects of RB1/pRb inactivation are species, tissues, and cell type specific

Inactivation of either RB1 or pRb, also determine effects which are species, tissues and cell type specific. For example, in contrast to retinoblastoma patients, in mice inheritance of one deleted copy of RB1 does not predispose to retinoblastoma, but to increased risk of pituitary and thyroid cancers (Williams et al., 1994a); deletion of both copies of RB1 results in ectopic proliferation, apoptosis and impaired differentiation in extraembryonic, neural, and erythroid lineages, culminating in foetal death by embryonic day 14.5 (E14.5; Wu et al., 2003); in trophoblast stem (TS) cells, but not in trophoblast derivatives of
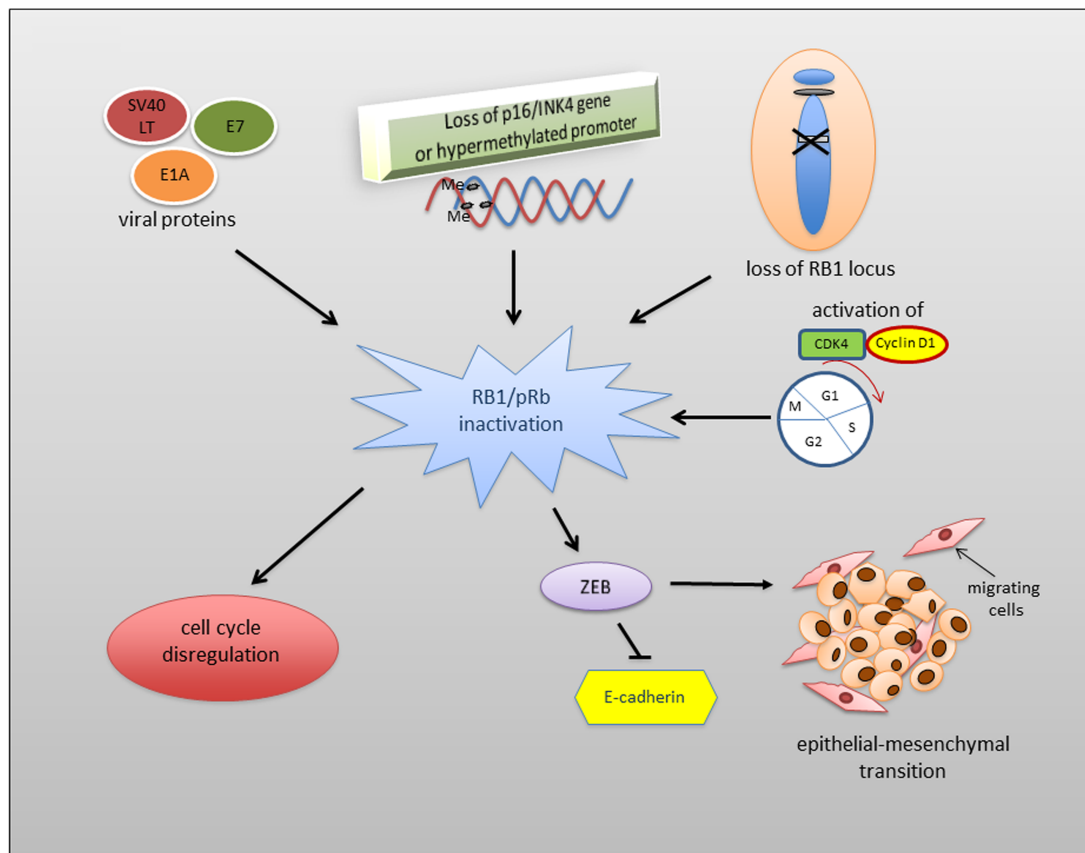
Fig. 2. Mechanism of RB1/pRb inactivation and its effects on cancer. Many different mechanisms can contribute to RB1/pRb inactivation: binding of viral oncoproteins (SV40 LT, E1A, and E7); loss of p16/INK4; p16INK4 promoter hypermethylation; deletion of RB1 locus; pRb phosphorylation/inactivation by CDK4/Cyclin D1. Exploration of molecular links between RB1/pRb inactivation and cancer revealed that loss of pRb function causes a disregulation of molecular brakes regulating cell cycle progression and an upregulation of ZEB protein, transcriptional repressor of the E-cadherin gene and key determinant of epithelial-mesenchimal transition.

the placenta, pRb is required for normal development (Wenzel et al., 2007). In these cells pRb would have a stem cell-specific function and its critical role would be mediated by E2F3. The specific loss of pRb in TS cells leads to an overexpansion of trophoblasts, a disruption of placental architecture, and fetal death by E15.5. pRb loss resulted in an increase of E2F3 expression, and the combined inactivation of pRb and E2F3 in TS cells restored placental development and extended the life of embryos to E17.5. Thus, the loss of pRb in TS cells would be the defining event causing lethality of pRb−/− embryos and the pRb pathway would play a critical role in the maintenance of a mammalian stem cell population. In addition, germline mutations in RB1 and TP53 is accompanied by pinealoblastomas, pancreatic islet cell tumors, bronchial epithelial hyperplasia and retinal dysplasia, suggesting that in mice these mutations can cooperate in the transformation of specific cell types (Williams et al., 1994b). Moreover, it seems that neoplastic transformation of human cells require more genetic changes than their murine counterpart; indeed, perturbation of two signaling pathways (involving p53 and Raf) suffices for the tumorigenic conversion of normal murine fibroblasts, whereas perturbation of six pathways (p53, pRb, PP2A, TERT, Raf, and Ral-GEFs) is needed for tumorigenic conversion of human fibroblasts (Rangarajan et al., 2004).

Differences were also evidenced in genetic alterations involved in lung cancer development, depending on whether

the tumors were neuroendocrine or not. More precisely, 87% of high-grade neuroendocrine lung carcinomas exhibit an abnormal expression of pRb that is highly correlated with a loss of heterozygosity at the RB1 locus. In contrast, in non-neuroendocrine carcinomas, pRb is mainly inactivated through loss of p16INK4a function and/or constitutive cyclin D1 activation. (Gouyer et al., 1998).

Moreover, there are tissues, as urothelial and retina in humans (He et al., 2009) and pituitary and thyroid in mice (Knudsen and Knudsen, 2006), that are significantly more susceptible than others to tumorigenesis arising with RB1 alterations. In these tissues, loss of pRb compromises critical facets of proliferation such that tumors readily arise from a field of aberrant cellular proliferation. In some tissue types, loss of pRb could abrogate differentiation programs, whereas in other tissues pRb loss would compromise genomic stability in a manner promoting tumorigenesis (Knudsen and Knudsen, 2006).

Overall, the development of malignancy, typically includes disruption of pRb activity through one of the many mechanisms that disrupt the p16INK4a–CDK4–cyclin D–pRb pathway. In melanomas pRb is inactivated through loss of p16INK4a; in retinoblastoma, prostate cancer and osteosarcoma, pRb is inactivated through direct mutation or loss of the RB1 locus (Nielsen et al., 1998); moreover, the alteration of key cell cycle regulators more frequently associated to RB1 loss seems to

affect p16INK4a (Indovina et al., 2010), whose downregulation was observed in 55% of retinoblastoma patients, and in 56% of these cases at least one of the parents bored the same alteration in blood cells. Additionally, in most patients with p16INK4a downregulation and in the parents with the same alteration, analysis of p16INK4a promoter showed hypermethylation (Indovina et al., 2010). Interestingly, 30% of human cancers that harbor gain-of-function in Ras show loss of p16INK4a expression, resulting in hyperphosphorylated/inactive pRb which plays important roles in maintaining the proliferative status of these cells (Williams et al., 2006). Thus, p16INK4a alteration could be a novel inheritable susceptibility marker to retinoblastoma and could open the way for the development of new preventive and therapeutic strategies using demethylating agents.

In human retinoblastoma Y79 cell line, the deregulated cell proliferation originated by the absence of pRb appeared to be strongly supported by both an authocrine loop IGFR/IGF1/IRS1-dependent (Giuliano et al., 1996), and a constitutive activation of Akt (D'Anneo et al., 2010). In the majority of lung cancers pRb is inactivated through loss of p16INK4a–cyclin D–CDK4/6–pRb pathway function. In particular, in the case of non-small cell lung cancer (Na-Hye, 2008) pRb inactivation can occur through distinct mechanisms including mutation, excessive CDK activation, deregulated phosphorylation through abnormal CDK4–cyclin D expression, and loss of p16INK4a activity by aberrant promoter methylation or homozygous deletions, or point mutations. In human esophageal cancer and in small cell lung carcinoma (Yokota et al., 1988) the inactivation of pRb is more frequently dependent on altered RB1 mRNA. Deregulation of several genes involved in cell cycle control has been reported in classic Hodgkin lymphoma and an aberrant copy number of chromosome 9 with the loss of one or more p16INK4a loci was detected in several cases (Kim et al., 2006). Lack of pRb and p16INK4a was observed in over 50% of squamous cell carcinoma (SCC) of the tongue, the most common intraoral malignancy, accompanied by overexpression of cyclin D1 and a strong reciprocal relationship between pRb and p16INK4a expression (Bova et al., 1999). In contrast, in SCC of the oral cavity and pharynx related to HPV infection, where pRb is inactivated by E7 and this results in an upregulation of p16 expression, this overexpression has been correlated to favorable prognosis (Weinberger et al., 2006). Moreover, loss of RB1 function by loss of heterozygosity has been reported in glioblastomas, breast cancer, gastric carcinoma, renal carcinoma, laryngeal cancer. About glioblastoma (the most common and lethal primary malignant brain tumor), recently (Dunn et al., 2012) extensive multiplatform genomic characterization has provided a higher-resolution picture of the molecular alterations underlying this disease. Among the 601 genes analyzed, the most significant somatically mutated genes were TP53, PTEN, NF1, EGFR, RB1, PIK3R1, and PIK3CA. This analysis permitted the projection of identified alterations onto known pathways revealing the high incidence of p53, pRb and receptor tyrosine kinase (RTK)/Ras/phosphoinositide 3-kinase (PI3K) pathway dysregulation, and confirming previous work that had delineated lesions in these critical cascades. The studies show that glioblastoma represents several histologically similar yet molecularly heterogeneous diseases, which influences taxonomic classification systems, prognosis and therapeutic decisions.

Murine embryonic fibroblasts are readily transformed by the introduction of specific combinations of oncogenes; however, the expression of those same oncogenes in human cells fails to convert such cells to tumorigenicity. Using normal human and murine embryonic fibroblasts (Boehm et al., 2005) it has been shown that the transformation of human cells requires several additional alterations beyond those required to transform

comparable murine cells. The introduction of the c-Myc and H-RAS oncogenes in the setting of loss of p53 function efficiently transforms murine embryonic fibroblasts but fails to transform human cells constitutively expressing hTERT, the catalytic subunit of telomerase. In contrast, transformation of multiple strains of human fibroblasts requires the constitutive expression of c-Myc, H-RAS, and hTERT, together with loss of function of the p53, RB1, and PTEN tumor suppressor genes. These manipulations permit the development of transformed human fibroblasts with genetic alterations similar to those found associated with human cancers and define specific differences in the susceptibility of human and murine fibroblasts to experimental transformation.

In tumor tissues of patients with non-small-cell lung carcinoma (NSCLC), it has been observed (Akin et al., 2002) a loss of expression of p16INK4a and/or pRb in 72 out of 95 patients, with 70 of them showing inverse correlation. Loss of p16INK4a expression was found to be significantly greater in SCC than in adenocarcinoma cases, with pRb negative SCC cases having significantly shorter survival. These results suggested that disruption of p16INK4a/pRb pathway is frequently involved in NSCLC and that, in cases with SCC, pRb expression loss may predict clinical outcome.

An additional level of control on the phosphorylation and inactivation of the pRb seems to be dependent on the peptidylprolyl isomerase Pin1 (Rizzolio et al., 2013), which regulates tumor cell proliferation allowing the interaction between CDK/cyclin complexes and pRb through direct interaction with the spacer domain of the pRb protein, and in such a manner selectively boosting the switch from hypo- to hyper-phosphorylated pRb. In addition, as shown in human malignant glioma tissue and in Pin1 knockout mice, synergistically with PI3K and CDKs, Pin1 plays a critical role in sustaining the complete phosphorylation of pRb.

It has been suggested that pRb inactivation can also contribute to tumor progression by conversion to an invasive phenotype. Indeed, it has been observed (Arima et al., 2008) that loss or reduction of pRb expression in high-grade breast adenocarcinomas, and knockdown of pRb by small interfering RNA in MCF7 breast cancer cells, disrupts cell–cell adhesion and induces a mesenchymal-like phenotype that is implicated in the metastasis of primary tumors. This was strongly supported by the findings that in human breast cancer cells depletion of pRb induces downregulation of the adhesion molecule E-cadherin and thereby triggers the epithelial–mesenchymal transition. In addition, in RB1-inactive cells that exhibited a mesenchymal-like morphology and were highly invasive, ZEB proteins—transcriptional repressors of the E-cadherin gene—were markedly upregulated. Moreover, depletion of ZEB in RB1-inactive cells suppressed cell invasiveness and proliferation and induced epithelial marker expression (Arima et al., 2012). These results implicate ZEB in induction of the epithelial-mesenchymal transition, and suggest that the inhibition of epithelial mesenchymal transition would be a novel tumor suppressor function of pRb.

The complexity of these regulatory mechanisms is complicated by the different functions played by the RB family members which in some circumstances function analogously, while in other can play very distinctive roles (Genovese et al., 2006; Neganova and Lako, 2008). Of the three pocket proteins, p130 has the highest expression level in quiescent cells (G0; Litovchick et al., 2007); under these growth conditions pRb expression is low, but detectable in complex with E2Fs, while p107 is nearly undetectable. In arrested cells, E2F4 is sequestered by the p130 protein. As the cells pass the G1-to-S transition, the levels of pRb and p107 increase and E2F4 now associates with both of these regulators. Once the cells enter S phase, free E2F is composed of an equal mixture of E2F4 and E2F1 (Moberg et al., 1996). Overall, the evaluation of the

expression levels of pocket proteins throughout the cell cycle (Henley and Dick, 2012), suggest that while pRb works equally well in both cycling and quiescent cells, instead, p130 works exclusively in quiescent cells, and p107 works exclusively in cycling cells. Moreover, similarly to pRb, even p107 and p130 act in a cell-type-and tissue-specific manner. For example, in C33A cervical cancer cells, the "mullerian inhibiting substance" inhibits cell proliferation by the induction of p107 and p130, whereas similar effects are not played in OVCAR8 epithelial ovarian cancer cell line (Barbie et al., 2003). The proliferation of T98G human glioblastoma cells is inhibited by p130, but not by pRb and p107 (Claudio et al., 1996). It can be stated that p130 has growth suppressive properties similar to yet distinctive from those of pRb and p107 (Canhoto et al., 2000), and that reduced levels of p130 are a powerful negative prognostic factor in several malignancies, enclosed soft tissue sarcomas and Burkitt lymphoma (De Falco et al., 2007; Masciullo et al., 2008).

Activation of the Ras oncogenic pathway and/or inactivation of pRb pathway are involved in most human cancers. Interestingly (Williams et al., 2006), Ras-induced oncogenic transformation seems to depend on functional pRb as tumors that have Ras mutations, usually keep expression of a wild-type pRb. Indeed, fibroblasts lacking pRb are less susceptible to the oncogenic actions of Ras than wild-type cells, and activated Ras has an inhibitory effect on the proliferation of pRb-deficient human tumor cells. In contrast, fibroblasts deficient in p107 and p130 are more susceptible to Ras-mediated transformation than wild-type cells. Moreover, loss of pRb in tumor cells harboring a Ras mutation results in increased expression of p107, which strongly inhibits proliferation of these tumor cells. These findings that pRb and p107 have distinct roles in Ras-mediated transformation suggested a novel tumor-suppressive role for p107 in the context of activated Ras.

In distinct epithelia as mammary gland, brain choroid plexus and prostate, pRb/p107/p130 inactivation induces aberrant proliferation and apoptosis (Hill et al., 2005). However, in mammary gland and brain choroid plexus, apoptosis is p53 dependent and tumors progress with selective inactivation of p53 (Simin et al., 2004). Instead, the apoptosis induced in the suppression of prostate tumor growth is regulated by the tumor suppressor PTEN (Hill et al., 2005), which was found mutated in a large number of cancers. In particular, it seems that inactivation of pRb initiates prostate cancer with the establishment of selective pressures that lead to diminished PTEN function and tumor evolution. Thus, although the biological effect of pRb inactivation is similar in all three epithelial cell types, the pathways on which selective pressure is imposed is distinct.

RB1 deletion also shows a great impact on mitogen dependence, anchorage dependence and overall survival which are influenced by the oncogene milieu.

Recently, employing a model of conditional genetic deletion (RB1 loss and Ras-transformed cells) to decipher the effects related to disease progression, it has been suggested that the impact of RB1 deletion is dependent on the oncogene milieu, and can directly contribute to transformed phenotypes and response to therapeutic intervention (Dean et al., 2010). More precisely, RB1 deficiency predisposed c-Myc-expressing cells to cell death and reduced tumorigenic proliferation. In contrast, RB1 deficiency exacerbated the tumorigenic behavior of Ras-transformed cells either in the model system or in human tumor cell lines. In addition, the evaluation of the sensitivity to cell death with RB1 loss showed that, although under pharmacological activation of the p53 pathway these Ras-transformed-RB-deficient cells bypassed the G1-checkpoint, they were also highly sensitized to cell death.

Chellappan et al. (1992) have shown that inactivation of pRb may be also determined by the transforming proteins of DNA tumor viruses such as the SV40 LT-antigen and HPV-E7 which target pRb during cellular transformation. In particular, they showed that HPV-E7 protein and the SV40 LT-antigen can dissociate the E2F-pRb complex. Moreover, since they found that the E2F-pRb complex is absent in various human cervical carcinoma cell lines that either express the E7 protein or harbor an RB1 mutation, they suggested that the loss of the E2F-pRb interaction through this mechanism may be an important aspect in human cervical carcinogenesis, also suggesting that this dissociating action may initiate the oncogenic process in a manner analogous to the mutation of the RB1 gene. Importantly, functional inactivation of pRb by viral oncoprotein binding is usually occurring in many neoplasias such as cervical cancer, mesothelioma and AIDS-related Burkitt's lymphoma (Masciullo et al., 2008).

Overall, the results suggest that antigrowth signals directed to pRb and cell cycle, are blocked in the majority of human tumors, thus preventing cells from exiting the cell cycle and entering G0. In short, different tumors exhibit selective alterations of the pRb pathway, as overexpression of CDK4–6, inactivation of p16INK4a, increased expression of cyclin D1, mutation of CDK4 or direct mutational inactivation of RB1. Why different tumors preferentially select for alterations in one component over the other is still unknown.

pRb and E2F Family in Cancer

Increasing evidence suggests that transcription factors (TFs) can be usefully employed as markers for cancer, potential prognostic markers, and targets for drug therapy. Deregulation of TFs that control the G1-S transition by engaging E2F activity, has been frequently evidenced in the process of neoplastic transformation where E2Fs can act both dependently and independently of cell cycle regulation (Chen et al., 2009) and can also play crucial roles in timely activation of G1/S and G2/M genes involved in cell cycle progression (Bracken et al., 2004; Westendorp et al., 2012).

RB pocket proteins and E2F transcription factors

The mammalian E2F family has eight members (Chen et al., 2009; Lammens et al., 2009, with references herein) which, based on structure–function studies in vitro, have been subdivided both into activators (E2F1–3a) and repressors (E2F3b–8) members and into typical (E2F1–6) and atypical(E2F7–8) members (Fig. 3). E2F1–6 members regulate transcription of their target genes by bounding to their promoters as dimers with a dimerization partner (DP) protein. This is possible because they possess one N-terminal DNA-binding domain (DBD) which is followed by a dimerization domain allowing interaction with DP1, DP2, or DP3. Heterodimerization, which is mediated by the leucine zipper (LZ) and the marked box (MB) domains, is a prerequisite for sequence-specific binding of these E2F proteins to the target genes. Moreover, E2F1–5 function through pocket protein binding domain present in the carboxyl-terminals, whereas since E2F6 do not possess this domain, it acts as a negative regulator countering the activity of other E2F members (Gaubatz et al., 1998).

E2F7–8 bind to promoters as homodimers or heterodimers without DP. This is possible because they have a duplication of the DBD (DBP1 and DBP2) each containing DNA-binding and dimerization sites (Logan et al., 2004).

Pocket proteins/E2F complexes are dynamic and change upon progression through the cell cycle. Apparently, the only clear biochemical distinctions between pRb and the related p107 and p130 pocket proteins are their E2F-binding preferences. In particular, in vivo, under normal conditions, E2F1–3 bind exclusively to pRb (Lees et al., 1993), whereas E2F4 can associate with all pocket proteins (Moberg et al., 1996) and
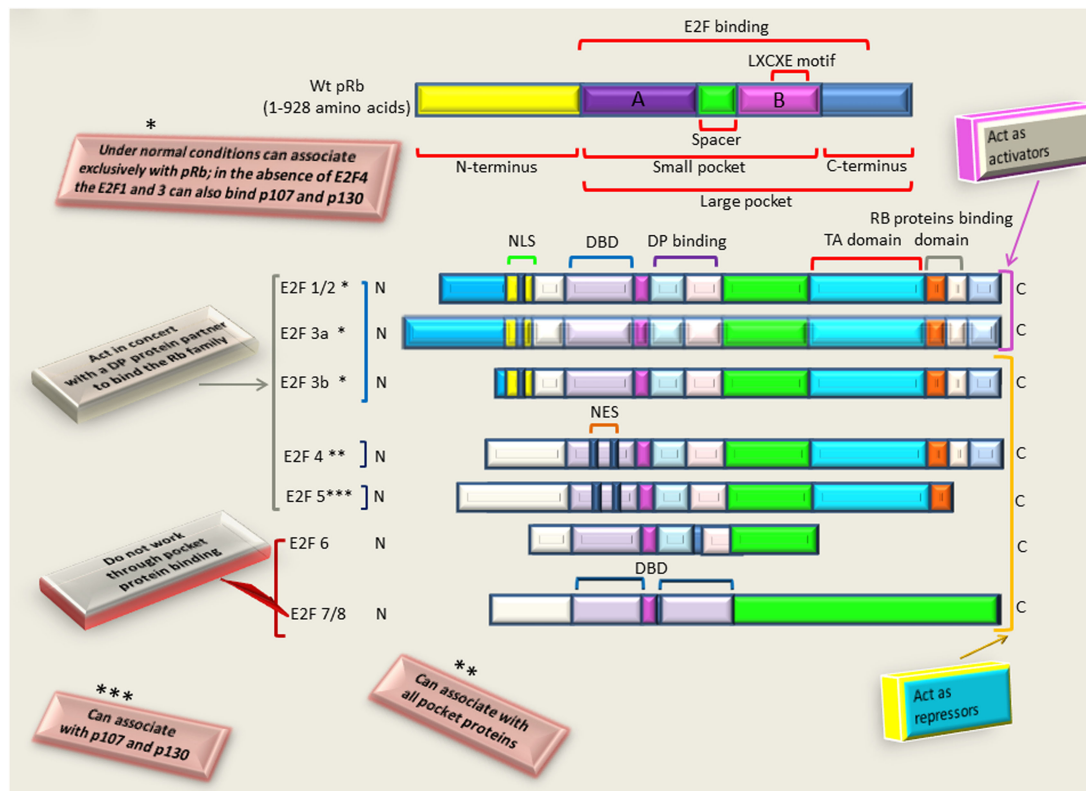
Fig. 3.   Crosstalk between E2F family proteins and pRb. Full-length pRb (amino acids 1–928) is depicted at the top with the large A/B pocket which was originally defined on the basis of its ability to interact with E2F transcription factors on DNA. E2Fs are a family of proteins that share a conserved DNA-binding domain (DBD) that binds to overlapping sets of target promoters. Eight E2F family members (E2F1–8) have been described. E2F1–6 contain a conserved dimerization domains and form DNA-binding heterodimers with proteins of the differentiation-regulated transcription factor-1 polypeptide (DP) family. Members of E2F1–5 contain C-terminal domains that mediate interactions with the pocket domains of RB-family proteins. E2F6–8 do not possess this domain. E2F1–3b share a nuclear localization signal (NLS) at their amino-terminal domains. Instead, E2F4 and E2F5 have a nuclear export signal (NES). E2F7 and E2F8 possess two DBD and do not have pocket protein binding domains. E2F1–3a seem to primarily function as transcriptional activators; E2F3b–8 seem to primarily function as transcriptional repressors.

E2F5 (although can also interact with p107) preferentially associate to p130 (Hijmans et al., 1995). However, in the absence of E2F4, the E2F1 and E2F3 activators, which normally do not bind p107 and p130, could now be recruited into a complex with these pocket proteins. The consequence of this pocket protein–E2F rearrangement is the sequestration of E2F activators into a protein complex that has a repressor function, raising the possibility that activators could compensate for repressors and vice versa in a context-dependent manner (Chen et al., 2009).

Roles of the E2F1–E2F3 members

In mice, the tumor suppressive properties of pRb are primarily dependent upon its ability to inhibit the activity of E2F1–3. Indeed, as shown by Chong et al., (2009), E2F1–3 play distinct roles in dividing versus differentiating cells and in normal versus cancer cell. In dividing progenitor cells, free E2F1–3 function as transcriptional activators, but, contrary to the current view, they are necessary for cell survival while are dispensable for cell division. Instead, in differentiating cells E2F1–3 act as repressors in a complex with pRb to silence E2F targets and facilitate cell cycle exit. In differentiating cells the inactivation of pRb results in a switch of E2F1–3 from repressors to activators, leading to ectopic cell divisions. Overall, the results suggest that, when associated with RB family members, E2F1–3 function as transcriptional repressors, whereas free E2F1–3 activates transcription. However, as mice lacking E2F1, E2F2, or E2F3 survive to mid gestation without global defects in the cell cycle, this suggests that under normal conditions E2Fs do not substantially contribute to the proliferative potential of a cell (Chen et al., 2009).

Deregulated E2F1 activity, as a consequence of pRb or p16INK4a inactivation or amplification of cyclin-D or CDK4, confers growth advantage to cancer cells and is a hallmark of human tumors (Wu and Yu, 2009). The study of Worku et al. (2008) demonstrated a compelling trend for lower level of expression of E2F1 gene in malignant versus normal tissues. Remarkably, it has been established a statistically significant inverse correlation between the expression of E2F1 genes and increasing TNM (Tumor, Node, Metastasis) stage of breast cancer. Such downregulation of E2F1 in malignant tissues suggests a tumor suppressive role for E2F1 in human normal tissues. In addition to cell proliferation control, E2F1 can also induce apoptosis under various cellular contexts. However, although it is well known that E2F1 is a proapoptotic factor, the mechanisms regulating its proapoptotic activity are not fully understood. In human retinoblastoma Y79 cells (Drago-Ferrante et al., 2008), the potent apoptotic effect induced by the anticancer drug paclitaxel (PTX) was accompanied by a potent

induction of E2F1 and was followed by G2/M arrest, and cyclines A, E, and B1 accumulation. This was also accompanied by a prolonged increase in p53 expression and stabilization, with its nuclear translocation and increases in the levels of its transcriptional targets (p21WAF1, bax, and MDM2). In human H1299 non-small lung carcinoma cells (Xie et al., 2006) E2F1 promoted p53-independent apoptosis through directly regulating Smac/DIABLO, its downstream mitochondrial apoptosis-inducing factors. Recently (Aoki et al., 2012), it has been shown that E2F family are covalently conjugated with NEDD8 a member of the ubiquitin-like protein family. Following DNA damage SENP8 (a cysteine protease that specifically cleaves NEDD8 from modified substrates) removed NEDD8 from E2F1. This strongly modified the target specificity of E2F1, enhancing its activity at a subset of target genes including p73. Thus, deNEDDylation has been suggested as a critical switch that directs E2F1 toward proapoptotic function. Moreover, Shi et al. (2011) have shown that resveratrol (a natural polyphenolic compound with cancer chemopreventive activity) induced apoptosis in breast cancer cells by E2F1-mediated upregulation of ASPP1, a new member of the ASPP (apoptosis stimulation protein of p53) family, which plays an important role in the regulation of apoptosis. Shortly, as p53 is frequently inactivated in human cancers, E2F1-induced apoptosis has been suggested as an additional tumor surveillance mechanism to protect the organism from tumor development.

Also concerning the E2F2 role, studies in mice and humans have suggested that it can function as a tumor suppressor. It has been shown that E2F2 transcriptionally represses cell cycle genes to establish the G0 state (Infante et al., 2008). Indeed disruption of the E2F2 gene causes T cells and murine embryonic fibroblasts to enter S phase early and to undergo accelerated cell division. E2F2 also functions as a tumor suppressor in epithelial tissues by limiting proliferation in response to Myc (Pusapati et al., 2010). In fact, in the skin and oral cavity, E2F2 inactivation cooperates with transgenic expression of Myc to enhance tumor development without affecting Myc-induced apoptosis. Pusapati et al. (2010) also show that E2F2 differentially regulates gene expression depending on the individual target in epidermal keratinocytes, thus confirming the critical importance of cell type specificity in cancer mechanisms.

E2F3 has emerged as a critical pRb-binding factor, important for mediating many of pRb functions during embryonic development and tumorigenesis. The E2F3 locus drives the expression of two related gene products, E2F3a and E2F3b, through the use of two distinct promoters. E2F3a protein accumulates maximally at the G1/S transition, whereas E2F3b protein is expressed in quiescent cells and remains constant throughout the cell cycle, this can explain the importance of E2F3 in mediating pRb function (Adams et al., 2000). E2F3 is a candidate bladder cancer oncogene, with overexpression of its isoforms (E2F3a and b) being evidenced in 6p22-amplified bladder cell lines (Hurst et al., 2008). However, in primary bladder tumors, the presence of hyperphosphorylated pRb form or the loss in p16INK4a suggested that, in addition to E2F3 overexpression, inactivation of the pRb pathway is required for these tumors. Recently it has been demonstrated that the strength of E2F3 expression is a potential marker for discriminating prostate benign and malignant disease (Pipinikas et al., 2007).

Roles of the E2F4–E2F6 members

E2F4 and E2F5 species are very poor transcriptional activators and they are unable to induce quiescent cells to enter S phase. They can form complexes with pRb, p107, and p130 and in this form can bind to E2F-responsive promoters and actively repress their transcription, thereby promoting quiescence (Dyson, 1998). E2F4, the most abundant E2F protein, has a transactivation domain but, due to the presence of strong nuclear export signals, in its free form is primarily localized to the cytoplasm and thus its transcriptional activity is restrained. In the G0/G1 phase of cell cycle E2F4, owing its interaction with the pocket proteins, accumulates in the nucleus where actively represses E2F-target (Gaubatz et al., 2001). E2F4 and pRb functionally interact in specific neuroendocrine tissues to restrain cell proliferation and it has been established a role for these proteins in the urogenital epithelium and in derivatives of neural crest cells (Parisi et al., 2009). In RB−/− E2F4−/− chimeric mice, loss of E2F4 differently affects the tumorigenicity of pRb-deficient tissues. The most striking effect were observed in the pituitary where E2F4 loss delays the development, and reduces the incidence of pRb mutant tumors (Parisi et al., 2009).

Regarding E2F5, Kothandaraman et al. (2010) investigated the involvement of E2F5 in the development of ovarian epithelial cancer (OEC), the most lethal gynecological malignancy in Western countries. Performing the study on a great number of women differently aged (20–72 years) with either normal, benign, or malignant tumors, they unambiguously showed that malignant tissues overexpress E2F5, while none of the normal and benign samples showed expression for E2F5. More precisely, E2F5 was found to be upregulated (fivefold) in early and late stage of ovarian tumors. As E2F5 falls in the E2Fs category of inhibitors of proliferation, the authors suggested that the increased E2F5 levels might be the result of an attempt of the body to arrest the proliferation of tumor cells during the early stage of the disease. The study suggests that high E2F5 levels in tissues and serum can be a potential marker for early OEC.

Recently, it has been shown that E2F5 positively regulates S-phase entry in HeLa cells and that this activation of the cell cycle is specific for HPV18-expressing cells. This suggested that HPV18 could act changing the role of E2F5 from being a cell-cycle repressor to an activator, thus contributing to the higher oncogenic potential of HPV18 respect to other high-risk HPV types (Teissier et al., 2010).

E2F6, similarly to the other E2F1–5 members, binds to E2F consensus sites, but in contrast to these members, it lacks an pRb binding domain and functions as an pRb-independent transcriptional repressors (Pohlers et al., 2005). Consistent with this finding, E2F6 can behave as a dominant negative inhibitor of the other E2F family members. The biological properties of E2F6 are mediated through its ability to recruit the polycomb transcriptional repressor complex which plays a well-established role in gene silencing (Attwooll et al., 2005). Oberley et al. (2003), using a combination of chromatin immunoprecipitation and genomic microarrays, have shown that many of the E2F6-regulated genes encode functions involved in tumor suppression and maintenance of chromatin structure.

In short, it is thought that genetic alterations resulting in the loss of pRb functions cause cancer by unleashing E2F activity and deregulating cell proliferation. However, given that a number of human tumors have concurrent pRb inactivation and E2F amplification and overexpression, it is possible that the E2F family can have alternative tumor-promoting activities independent of cell cycle regulation.

The atypical E2Fs family members E2F7 and E2F8

Recently, a novel evolutionarily conserved branch of E2F-related TFs has been discovered which, owing to their peculiar structural properties, were designated atypical E2F proteins. As described by Lammens et al. (2009), these atypical E2Fs family members (E2F7 and E2F8), have a duplicated DBD and control gene expression without heterodimerization with partner

proteins. They are involved in post-mitotic development, embryogenesis and carcinogenesis also playing crucial functions during development (control of cell size, endocycle, proliferation and DNA-damage response). Given their crucial role in controlling proliferation, proper mitotic entry and DNA-damage induced apoptosis they might function as putative tumor suppressors in the tumor setting. In addition (Moon and Dyson, 2008) it seems that these proteins, together with E2F1, form a cell-cycle-dependent feedback loop in which the transcription of E2F7 and E2F8 increases at the G1/S phase transition along with other E2F targets. Then, as E2F7 and E2F8 accumulate, in turn repress the transcription of E2F1, limiting the level and window of E2F1 activity. Additionally, Li et al. (2008) have shown that for mice cell survival and embryonic development, it is essential that E2F7 and E2F8 synergize.

In ovarian tumor tissues low levels of E2F7 have been associated with low patient survival and potential development of resistance to anticancer drugs (Reimer et al., 2007). Recently, Westendorp et al. (2012) employing the combination of two independent genome-wide approaches (ChIP-seq technology and short term induction of E2F7), identified 89 target genes that carry E2F7 binding sites close to the transcriptional start site and that were directly repressed by short-term induction of E2F7. These genes, which were considered as the top E2F7 target genes, encode gene products involved predominantly in DNA replication, metabolism and DNA damage response (RAD51, CHEK1, and BRCA1−2 genes). Since the transcription of the majority of the E2F1−3a target genes is repressed directly by E2F7, the authors suggested that E2F7 counterbalances the transcription of E2F target genes activated by E2F1−3a. They also showed that protein levels of E2F7 accumulate maximally at mid to late S-phase coinciding with the time when the expression of E2F target genes declines. Conclusively, they stated that classical E2F activators are essential for the upswing in the oscillating pattern of the cell cycle-specific expression of G1/S genes, whereas the atypical E2Fs contribute directly to the downswing. In sharp contrast to E2F7, high levels of E2F8 are correlated with poor survival in glioma patients (Lammens et al., 2009). Moreover, Deng et al. (2010) have reported that E2F8 is strongly upregulated in human hepatocellular carcinoma (HCC), where it contributes to oncogenesis and progression and have suggested that E2F8 contributes to the oncogenic potential of HCC, thus constituting a potential therapeutic target in this disease.

Conclusions

The mechanisms of RB/pRb inactivation and the loss of pRb/E2F interaction found in cancer evidence that the intervention of these molecules in cell cycle control is a basic event to avoid cancer and that these inactivating mechanisms cause cancer by unleashing E2F activity. However, given that a number of human tumors have concurrent pRb inactivation and E2F overexpression, it is possible that the E2F family can have alternative tumor-promoting activities independent of cell cycle regulation, so how it is possible that pRb can play its regulatory roles independently of E2F family. Importantly, the effects of RB1/pRb inactivation are species, tissues and cell type specific, and the complexity of these regulatory mechanisms is complicated by the different functions played by the RB and E2F family members. Anyway, the deregulation of the roles played by RB proteins and E2Fs leads to cancer development. It seems that the majority of human tumors can block all the antigrowth signals directed to pRb and cell cycle, thus preventing cells from exiting the cell cycle and entering G0. Moreover, although the reason is still unknown, it is well known that different tumors preferentially select for alterations in one component over the other. Thus, despite the nearly 6,000 papers published on RB in cancer, more studies are needed to understand how pRb can

contribute to cancer origin and development. This may lead to design novel therapeutic strategies.

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