Minireview

Cyclin-dependent kinases and pRb: regulators of the proliferation-differentiation switch

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Abstract The retinoblastoma susceptibility gene (*RB1*) is essential for normal embryonic development. Loss of *RB1* leads to uncontrolled proliferation of a number of cell types but may also prevent proper terminal differentiation. The growth-suppressive and differentiation-inducing properties of pRb are impaired by cyclin-dependent kinase (cdk)-mediated phosphorylation. Hence, inhibition of cdk activity is probably a prerequisite for terminal differentiation. Indeed, forced cyclin or cdk expression can prevent terminal differentiation in various cell types, probably through inhibition of pRb and, possibly, differentiation-specific transcription factors.

Key words: Retinoblastoma gene product; Differentiation; Tumorigenesis; Cyclin-dependent kinase

1. Introduction

The retinoblastoma susceptibility gene (*RB1*) is the prototype tumour suppressor gene. Inactivating mutations in both alleles are required for the induction of tumorigenesis. In man, germline mutations in one *RB1* allele give a predisposition to retinoblastoma and, to a lesser extent, osteosarcoma. In the resulting tumours the remaining allele is inactivated indicating that loss of *RB1* is rate-limiting for tumorigenesis (reviewed in [1]). Inactivation of *RB1* as a result of somatic mutations is also frequently observed in a variety of other tumours. In these tumours loss or Rb function apparently leads to a proliferative advantage, but is not involved in the initiating step of tumorigenesis [1]. The observation that re-introduction of wild-type (wt) *RB1* into a number of *RB1*-negative tumour cell lines results in cessation of cell proliferation and loss of tumorigenicity, classifies *RB1* as a bona fide tumour suppressor gene [1].

The molecular mechanism by which the *RB1*-encoded protein, pRb, inhibits cell growth is becoming increasingly clear. pRb binds to and inhibits the action of various proteins, including the ubiquitously expressed tyrosine kinase c-Abl, the T-cell-specific transcription factor Elf-1 and members of the E2F family of transcription factors [2–4]. These proteins function by stimulating progression through S-phase. Cell cycle-dependent phosphorylation of pRb by cdk's releases these proteins from pRb-imposed inhibition and ensures proper timing of their activities [2–4]. Thus, pRb inhibits cell growth by preventing initiation of the S-phase. In addition to its function as a cell cycle regulator, recent work has established a role for pRb in mediating the terminal differentiation of a number of cell types.

2. pRb as a regulator of terminal differentiation

In an attempt to assess the function of pRb in vivo, several groups have generated mouse strains carrying inactive copies of the *RB1* gene [5–7]. Heterozygosity for *RB1* does not lead to an overt developmental phenotype, but homozygous loss of *RB1* results in embryonic lethality. Embryos develop normally until day 11 of gestation, after which gross abnormalities in neurogenesis and erythropoiesis occur, leading to embryonic cell death around day 15 [5–7]. The abnormalities occur in tissues where expression of the *RB1* gene is highest during development [8]: in neural tissue and in the fetal liver, the site of erythropoiesis at that stage of development. When the defects in neurogenesis were examined in more detail, it was observed that at sites where normally only post-mitotic cells are found, many cells attempt to divide and undergo apoptotic cell death [9]. Apparently, loss of pRb leads to uncontrolled proliferation followed by cell death, resulting in degeneration of the central and peripheral nervous systems. In addition to uncontrolled proliferation and apoptotic cell death a second phenomenon was observed in $Rb^{-/-}$ neural tissue: the surviving sensory neurons are less differentiated than their wild-type counterparts, as demonstrated by the lack of expression of neurotrophic factor receptors [9]. Signalling through these receptors is normally required for proper terminal differentiation and survival of neuronal cell types. These observations already suggest that impaired differentiation is not merely resulting from a failure to arrest the cell cycle, but that pRb also acts to establish terminal differentiation and to ensure cell survival. Indeed, a number of $Rb^{-/-}$ differentiation-committed post-mitotic neuronal cell types express early differentiation markers, yet fail to mature into fully differentiated neurons [9,10]. Second, $Rb^{-/-}$ lens fibre cells express early, but not late differentiation markers, suggesting a function for pRb during more advanced stages of the differentiation process [12]. In addition to neuronal disorders, $Rb^{-/-}$ embryo's suffer from impaired erythrocyte differentiation [5–7]. However, studies on the phenotype of chimaeric $Rb^{+/+}/Rb^{-/-}$ mice showed that this was an indirect effect of liver malfunction, and that pRb is not essential for terminal differentiation of erythrocytes as such, but may be required for proper functioning of hepatocytes [10,11].

The chimaeric $Rb^{+/+}/Rb^{-/-}$ mice developed normally, even though the contribution of $Rb^{-/-}$ cells to all tissues examined, including neural tissue, was considerable [10,11]. This suggested

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that loss of *RB1* is largely rescued by the presence of wt cells, suggesting that pRb is not required cell-autonomously. However, the fact that enhanced levels of cell death were found in the spinal ganglia, the retina, and the eye lens of the chimaeric animals [10–12], suggests that selection for $Rb^{+/+}$ cells is taking place in these tissues. Indeed, the retina of the chimaeric animals consisted mainly of $Rb^{+/+}$ cells [11]. Thus, pRb is required cell-autonomously in a restricted number of cell types. The neuronal ($Rb^{-/-}$) cell types that were not affected in the chimaeric animals may be rescued by the presence of neighbouring $Rb^{+/+}$ cells. Although the existence of such a trans-acting rescue mechanism has not been proven, it may involve secretion of signalling proteins with differentiation-inducing properties. The molecular mechanism through which pRb mediates terminal differentiation remains to be elucidated and is possibly cell type dependent.

In summary, loss of Rb function has, either directly or indirectly, profound effects on the terminal differentiation of various cell types in vivo.

In vitro studies on the terminal differentiation of myoblasts into myotubes have implicated pRb in the regulation of this process [13]. Underphosphorylated pRb mediates the formation of a heterodimeric transcription factor composed of MyoD (or its relatives myogenin or Myf5) and E2 [13]. This transcription factor complex drives muscle-specific gene expression and its activation is sufficient to convert some non-myogenic cell types into myogenic cell types [14]. In terminally differentiated myotubes pRb is underphosphorylated and thus constitutively active as a growth suppressor and as an inducer of myogenic gene expression. Why then, if pRb is so important in the regulation of this process, do the $Rb^{-/-}$ embryos not display any muscular abnormalities? The answer to this question has recently been provided: pRb belongs to a (still small) family of proteins with growth suppressing properties, the other family members being p107 and p130. When p107 was tested for its ability to activate MyoD-dependent gene expression, it appeared that it is as capable as pRb in performing this task [15]. In addition, when $Rb^{-/-}$ myoblasts are stimulated to differentiate, an increase of p107 expression is seen in the $Rb^{-/-}$ but not in the wt cells, strongly suggesting that p107 substitutes for pRb in the regulation of terminal myogenic differentiation [15]. However, it also implies that the function of pRb may be much more pleiotropic than the phenotype of the $Rb^{-/-}$ mice would suggest. Both p107 or p130, or even other unidentified family members, may substitute for pRb in the regulation of differentiation or other processes, thereby masking the fact that normally pRb would mediate these processes.

3. pRb as a tumour suppressor

Mice heterozygous for *RB1* develop normally but are predisposed to pituitary tumours, rather than retinoblastomas [5,6,10,11]. In the pituitary tumours the remaining wt allele is lost. This, together with the observation that chimaeric $Rb^{+/+}$/$Rb^{-/-}$ mice develop pituitary tumours at much earlier age than the $Rb^{+/-}$ mice [10,11], suggests that loss of *RB1* is a rate-limiting step in the genesis of pituitary tumours in the mouse, as it is for retinoblastomas in man. The control of cell cycle progression in these cell types is thus largely dependent on pRb, and loss of its function is apparently sufficient to initiate tumorigenesis. The ubiquitous expression pattern of *RB1* in adult

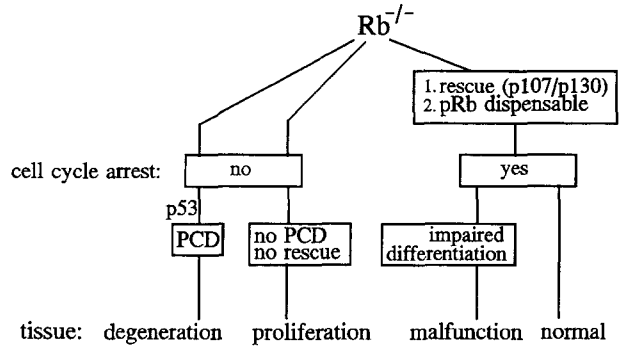

Fig. 1. Effects of *RB1*-loss on cell proliferation and differentiation. Homozygous loss of *RB1* has different effects on different cell types. In a number of cell types loss of pRb leads to loss of cell cycle control, followed by p53-induced programmed cell death (PCD), resulting in tissue degeneration. In cells where uncontrolled proliferation does not lead to PCD, initiation of tumorigenesis can occur. Alternatively, loss of pRb may contribute to, but not be sufficient for, the initiation of tumorigenesis. Loss of cell cycle control may also be rescued by pRb-related proteins, ensuring cell cycle arrest. In some cell types where pRb is not required for cell cycle arrest, or where its function is rescued, loss of pRb may affect the ability of cells to undergo terminal differentiation, possibly resulting in tissue malfunction. See text for details and references.

tissues and the characterisation of pRb as a constrainer of cell growth seems contradictory to the limited range of tumour types that is directly correlated with loss of its function. A simple explanation could be that, although present in many cell types, pRb is not required for cell cycle control in most of these cell types and loss of its function will thus remain without effect. Alternatively, in certain cell types loss of *RB1* may contribute to tumorigenesis, but additional genetic changes may be required for the initiation of tumour growth. Another possibility is that p107 or p130 may substitute for pRb as growth suppressors. Although aberrations in the chromosomal region harbouring the p107 gene are not observed in common forms of cancer [16], the region harbouring the p130 gene is lost in several tumour types [17]. Finally, in $Rb^{-/-}$ mice, degeneration of a number of tissues was observed, apparently due to apoptotic cell death [5–7], suggesting that in these cell types tumorigenesis is prevented by activation of the apoptotic pathway. Recent studies have implicated p53, another tumour suppressor protein, in this process. In a p53 nullizygous background the tumour-take in response to heterozygous loss of *RB1* is greatly enhanced [18]. Furthermore, inactivation of pRb and its family members by targeted expression of viral oncogenes in specific cell types (including retina cells) results in apoptotic cell death and slow or no tumour growth in the presence of p53. However, in the absence of functional p53, loss of *RB1* leads to uncontrolled proliferation in these cells [12,19–22]. Thus, in a number of cell types, including retinoblasts, tumorigenesis as a result of loss of *RB1*, is prevented by the activation of a p53-mediated mechanism that leads to apoptotic cell death. Although these studies provide an explanation for the fact that $Rb^{+/-}$ mice are not predisposed to retinoblastoma [12,21,22], the question remains why human retina cells and mouse pituitary cells are sensitive to the loss of pRb function, as consecutive loss of p53 is rarely observed in these tumours [1,18]. In Fig. 1 the possible fates of cell types having lost *RB1* is schematically outlined.

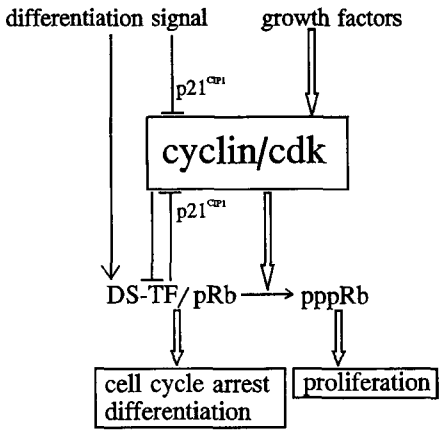

Fig. 2. Control on cdk activity during the proliferation-differentiation switch. The model is based on reports describing terminal myogenic differentiation and is hypothetically generalised. In response to a differentiation signal, CKI-expression (possibly $p21^{CIP1}$) is stimulated, resulting in an inhibition of cdk activity. With this, the inhibitory phosphorylations on pRb and differentiation-specific transcription factors (DS-TF) are lost. As a result pRb arrests the cell cycle and, in conjunction with the DS-TF, helps establishing the genetic program leading to terminal differentiation. The DS-TF locks cells in a growth factor non-responsive state by stimulating expression of the genes encoding $p21^{CIP1}$ and pRb. Possibly, additional pathways (DS-TF's) are activated in response to the differentiation signal to ensure terminal differentiation.

4. G1 cdk's as regulators of the proliferation-differentiation switch

Several years ago it was observed that phosphorylation of pRb is lost during terminal differentiation of various cell lines in vitro [23,24]. Moreover, underphosphorylated pRb accumulates in terminally differentiated neurons [9,25]. Specifically the underphosphorylated form of pRb binds to c-Abl, Elf-1, E2F's and MyoD, implying that inhibition of pRb phosphorylation will activate both its growth suppressive and (myogenic) differentiation-inducing potential. As mentioned in the introduction, cyclin-dependent kinases are believed to function as pRb kinases. Of the cyclin-cdk complexes, cyclin D-cdk4/6 and cyclin E-cdk2 presumably cooperate to induce hyperphosphorylation of pRb during late G1, prior to the onset of DNA synthesis [2]. Therefore, it seems likely that inhibition of cdk activity is a prerequisite for the switch from proliferation to differentiation to occur. Multiple levels of regulation determine the activity of a cdk [26]. Activation requires association to a cyclin partner and phosphorylation of the kinase itself. Inhibition of cdk activity also involves phosphorylation and, in addition, association to cdk-inhibitors (CKI's). During various terminal differentiation pathways, the expression of both cyclin as well as cdk subunits were shown to be prone to downregulation, precluding the formation of active cyclin/ckd complexes [27–30]. In addition, enhanced expression of the CKI $p21^{CIP1}$ has recently been observed in a number of differentiation pathways [31–34]. During embryonic development, the highest levels of $p21^{CIP1}$ expression are confined to post-mitotic cells of various origins [34]. During terminal myogenic differentiation the genes encoding $p21^{CIP1}$ and pRb are direct targets for transcriptional activation by MyoD [33,35], presenting an explanation for growth suppression by MyoD, even in non-convertable cell types. In addition, the expression of $p21^{CIP1}$ during myogenesis in vivo coincides with that of myogenin, suggesting that myogenin may be the physiological regulator of $p21^{CIP1}$ expression during myogenesis [34]. It has even been suggested that $p21^{CIP1}$ overexpression may be sufficient to induce myogenesis in vitro, although this process seemed to require contact-inhibition [36]. Another CKI, $p27^{KIP1}$, has been reported to be activated in response to contact-inhibition [37], so may help to establish terminal differentiation.

When terminally differentiated myotubes are stimulated with growth factors, pRb phosphorylation does not occur and cells do not synthesize DNA. Thus, terminally differentiated cells are locked in a state in which they are non-responsive to growth factor stimulation. The presence of high levels of CKI's, inhibiting cdk activity and pRb phosphorylation, may (partly) explain why terminally differentiated cells are refratory to growth factor stimulation.

In another approach to studying the importance of cdk regulation during differentiation, various groups have investigated the effects of cyclin or cdk overexpression on the commitment of a cell to undergo terminal differentiation [27–29]. In response to a differentiation signal, mouse myeloid precursor cells overexpressing either cyclin D2 or D3 do not differentiate but die [28]. Overexpression of the cyclin D-dependent kinase cdk4, but not cdk2 inhibits erythroid differentiation in vitro [29]. As pRb function is not required for erythroid differentiation in vivo (see above) a cdk4-mediated effect other than pRb phosphorylation presumably underlies this phenomenon. Evidence that cyclin D-dependent kinases indeed control the activity of additional regulatory proteins was recently presented: in myoblasts, cyclin D-dependent kinase activity was found to prevent MyoD-dependent promoter activation [27,36]. This effect is a result of cyclin D1-mediated MyoD-phosphorylation, resulting in its inactivation. Moreover, specific inhibition of cyclin D-dependent kinase activity by overexpression of the CKI $p16^{INK4}$ stimulates MyoD-dependent promoter activation, indicating that cyclin D-dependent kinases may serve to keep the differentiation program silent by inactivating both pRb and differentiation-inducing transcription factors [36]. It is tempting to extrapolate these findings and to also postulate the existence of master class transcription factors for other differentiation routes, being prone to negative regulation by cyclin (D)-dependent kinases (Fig. 2). In this respect, it must be noted that although all three D-type cyclins activate cdk4 and cdk6 there are major differences with respect to both their expression patterns [38,39] and functional performances [27,28,36,40,41]. For instance, the abundance of cyclin D3, but not D1 or D2, is increased during both terminal myogenic [30,36] and erythroid [29] differentiation. During these processes cyclin D3 accumulates in inactive kinase complexes with unknown (if any) functional consequences. In addition, cyclins D2 and D3 can activate cdk2 whereas cyclin D1 can not [41]. Conversely, a mutant form of cyclin D1 that is unable to bind pRb is more effective in relieving pRb-induced cell cycle arrest than wt cyclin D1, whereas a similar D2 mutant is ineffective [40,41]. Given these differences it is impossible to assign a universal function to the D-type cyclins in differentiation or cell cycle control.

An effect of cyclin E overexpression on terminal differentiation has not been reported. It seems likely however, that, if possible to achieve, artificially sustained cyclinE-cdk2 activity

would suffice to prevent pRb-dependent terminal differentiation.

In summary, the switch from proliferation to differentiation involves inactivation of cdk activity. As a result phosphorylation of pRb is lost, leading to activation of its function as a growth suppressor and inducer of differentiation. Additionally, the loss of cdk activity may lead to activation of differentiation-specific transcription factors.

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